IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:

MEHLHORN, Rolf Joachim

Serial No.: 10/759,222

Filed: 20 January 2004

For: METHOD FOR LOADING LIPID LIKE VESICLES WITH DRUGS OR OTHER CHEMICALS

Group Art Unit: 1614

Examiner: Kevin E. Weddington

Mail Stop Appeal Brief-Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPEAL BRIEF

Dear Sir:

This appeal brief is submitted in support of the Notice of Appeal filed on 30 September 2009.

REAL PARTY IN INTEREST

The real party in interest is the Regents of the University of California, having its primary place of business at 1111 Franklin St., 12th floor, Oakland, CA. This application is a continuation of Application Serial No. 08/472,843, filed 7 June 1995. The '843 application was itself a divisional application of Application Serial No. 07/741,305, filed on 7 August 1991. The original assignment to the Regents was recorded at Reel/Frame 4757/0030-0032 on 25 October 1994.

RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences that might have any bearing, direct or indirect, on the Board's decision in this appeal.

STATUS OF CLAIMS

The current status of the claims is:

Claims 1-26 are pending.

Claims 13-15 and 24-26 have been withdrawn by the examiner.

Claim 1-12 and 16-23 stand rejected are the subject of this appeal.

STATUS OF AMENDMENTS

All amendments have been entered. The claims were last amended on 27 May 2008 in response to a non-final office action mailed 29 November 2007.

SUMMARY OF CLAIMED SUBJECT MATTER

This invention is directed to a simple yet elegant means of loading lipid-like vesicles with a chemical where, after loading, the concentration of the chemical is greater inside the vesicle than outside the vesicle yet the gradient can be maintained for at least one quarter hour following loading. That is, the claims on appeal include four independent claims, claims 1, 2, 16 and 20. Claim 1 is directed to:

> 1. A method of loading lipid-like vesicles, comprising:
>
> forming lipid-like vesicles in a solution comprising an acidic buffer if the chemical species to be loaded is basic or a basic buffer if the chemical species to be loaded is acidic, wherein:
>
>> membranes of the formed lipid-like vesicles are impermeable to the buffer;

adjusting the pH of the solution exterior to the membranes of the lipid-like vesicles to a basic pH if the chemical species to be loaded is basic or to an acidic pH if the chemical species to be loaded is acidic;

adding a basic chemical species to the adjusted basic exterior solution or an acidic chemical species to the adjusted acidic exterior solution;

loading the chemical species into the vesicles; and

adjusting the exterior solution to a physiologically benign pH, wherein:

the chemical species is substantially maintained in the vesicles for at least one quarter hour after the adjustment of the exterior solution.

Support for "lipid-like" vesicles can be found at least on page 3, paragraph [0023] of published patent application serial number US 2004/0208922, which is the published version of the instant application.

Support for forming lipid-like vesicles in solutions comprising a buffer wherein the acidity/basicity of the buffer is dependent on the acidity/basicity of the chemical to be loaded into the vesicles can be found at least on page 4, paragraph [0028].

Support for membranes of the lipid-like vesicles being impermeable to the buffer can be found at least on page 4, paragraph [0031].

Support for adjusting the pH of the solution exterior to the vesicles can be found at least on page 4, paragraphs [0027] and [0028].

Support for adding a basic or acidic chemical species to the adjusted exterior solution can be found at least on page 4, paragraph [0030].

Support for loading the chemical species into the vesicles can likewise be found at least on page 4, paragraph [0030].

Support for adjusting the exterior pH to a physiologically benign pH after the chemical species is loaded can be found at least on page 4, paragraph [0030].

Support for the chemical species concentration being maintained for at least 15 minutes can be found at least on page 4, paragraph [0033].

Independent claim 2 is directed to:

2. A method of loading lipid-like vesicles having a membrane permeable to a chemical species to be loaded and for substantially maintaining the loaded chemical species within the vesicle for at least one-quarter hour following loading by inducing a pH gradient across the membrane, comprising:

(1) incorporating within the vesicle a buffer solution buffered to a selected acid or alkaline pH having a selected molarity and at last one selected pK_a approximately equal to the selected buffer pH, the membrane being substantially impermeable to the buffer for at least one-quarter hour following loading of the chemical species;

(2) positioning the vesicles in a bulk solution having a selected pH, and

(3) providing the bulk solution with a chemical species having one or more selected acid pH responsive groups if the buffer is alkaline or one or more basic pH responsive groups if the buffer is acidic wherein the pH of the bulk solution is at least 0.5, 0.3 or 0.2 of a pH unit higher than the pH of the buffer of the buffer is acidic and the chemical species has one, two, or three or more basic pH responsive groups, or the pH of the bulk solution is at least o.5, 0.3, or 0.2 of a pH unit lower that the pH of the buffer if the buffer is basic and the chemical species has one, two or three or more acid pH responsive groups, the pH responsive groups of the chemical species having one or more acid pH responsive groups have a pK_a that is generally lower than or equal to the pH of the bulk solution and generally higher than or equal to 3.5 and the pH responsive groups of the chemical species having one or more basic pH responsive groups have a pK_a that is generally higher or equal to the pH of the bulk solution and generally lower than or equal to 11.

Support for incorporating within a vesicle a buffer solution buffered to a selected pH, molarity and pK_a can be found throughout the application, in particular in paragraphs [0025], [0026], [0027], [0028], [0029], [0030] and [0031].

Support for positioning the vesicles in a bulk solution having a selected pH can be found at least in paragraph [0026].

Support for providing the bulk solution with a chemical species having acid or alkaline pH responsive groups can be found at least in paragraphs [0010] and [0026].

Support for having the pH of the bulk solution at least 0.5, 0.3 or 0.2 units different from the pH of the buffer within the vesicles can be found at least in paragraphs [0010] and [0011].

Independent claim 16 is directed to:

> 16. A kit for loading lipid-like vesicles having a membrane permeable to the chemical species to be loaded comprising:
>
> (1) a first compartment having a first solution having membranous lipid-like vesicles incorporating a buffer buffered to a selected acid or basic pH, the buffer having at least one selected pK_a approximately equal to the selected buffer pH and a selected molarity and being substantially impermeable to the vesicle's membrane for at least one-quarter hour following loading of the chemical species and the first solution having a selected pH such that the stability of the vesicle and its buffer can be maintained for a period of at least one week at 4 °C;
>
> (2) a second compartment, separate from the first compartment, having a second solution at a selected pH;
>
> (3) a chemical species permeable to the vesicle having a selected pK_a and one or more selected acid pH responsive groups if the buffer is basic or one or more basic responsive groups if the buffer is acidic, the chemical species being initially present in a selected one of two solutions with the second solution having a pH such that the mixture of the first and second solutions would have a pH at least 0.5, 0.3 or o.2 of a pH unit higher than the pH of the buffer if the buffer is acidic and the chemical species has one, two, or three or more basic pH responsive groups at least 0.5, 0.3 or 0.2 of a pH unit lower than the pH of the buffer if the buffers is basic and the chemical species has one, two or three or more acid pH responsive groups, the pH responsive groups of the chemical species having one or more acid pH responsive groups hav a pK_a that is generally lower than or equal to the pH of the mixture of the first and second solution and generally higher than or equal to 3.5 and the pH responsive groups of the chemical species having one or more basic pH responsive groups have a pK_a that is generally higher than or equal to the pH of the mixture of the first and second solutions and generally lower than or equal to 11.

Support for all the elements of claim 16 can be found on at least page 2, paragraph [0013].

Independent claim 20 is directed to:

> 20. a kit for loading lipid-like vesicles having a membrane permeable to an acid or basic chemical species to be loaded comprising:
> (1) a first compartment having a first solution having membranous lipid-like vesicles incorporating a buffer buffered to a selected basic pH if the chemical species to be loaded is an acid or acid pH if the species is a base, the buffer having a selected pK_a and a selected molarity, the membrane being substantially impermeable to the buffer for at least one-quarter hour following loading of the chemical species, the first solution having a selected pH such that the stability of the vesicle and its buffer will be maintained for a period of at least one week at 4 °C;
> (2) a second compartment having a first substance which when combined with the first solution will adjust the pH of the first solution so as to provide a predetermined pH gradient between the buffer within the vesicle and the pH adjusted first solution; and
> (3) a third separate compartment having a second substance which when combined with the pH adjusted first solution with further change the pH of said solution to a physiologically benign value with regards to the blood of a mammal.

Support for all of the elements of claim 20 can be found on at least page 2, paragraphs [0013], [0014] and [0015].

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The grounds for rejection to be reviewed in this appeal are:

1. Whether claims 1-8, 10-12 and 16-23 contain subject matter that was not described in the specification is such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention (written description rejection).

2. Whether claims 1-12 are unpatentable over Nichols, et al., Catecholamine Uptake and Concentration by Liposomes Maintaining pH Gradients, Biochimica et Biophysica Acta, 1976, 455:269-271 (Evidence Appendix, Exhibit "A"), Deamer, et al., The Response of Fluorescent Amines to pH Gradients Across Liposome Membranes, Biochimica et Biophysical Acta, 1972, 274:323-335 (Evidence Appendix, Exhibit "B"), or

Cramer, et al., NMR Studies of pH-Induced Transport of Carboxylic Acids Across Phospholipid Vesicle Membranes, Biochemical and Biophysical Research Communications, 1977, 75:295-301 (Evidence Appendix, Exhibit "C").

ARGUMENT

Claims 1-8, 10-12 and 16-23 are fully supported by the specification in their claimed breadth and meet the requirements of 35 U.S.C. § 112, first paragraph, with regard to written description.

Simply put, the examiner demands too much, certainly far more than required by extant law as it should be applied in this case. The examiner has maintained the same § 112 rejection, in essentially the same language throughout prosecution of this application, q.v. Office Action mailed 5 September 2008 (OA1, Evidence Appendix Exhibit "D"), Office Action mailed 31 March 2009 (OA2, Evidence Appendix, Exhibit "E"). The examiner's first argument is entirely misplaced in that it attempts to equate disclosing a "laundry list of every possible moiety" which was previously found to not constitute an adequate written description in Fujikawa v. Wattasasin, 93 F.3d 1559 (Fed Cir. 1996) with appellant's disclosure of extremely specific genera of chemical species, *i.e.*, those bearing acid or base responsive groups (Published Patent Application 2004/0208922, Exhibit "F", paragraphs [0008], [0009], [0010], [0011], [0022], [0023] and [0024] at least and claim 1, *supra*) and the detailed disclosure of specific relationships between the pHs of the various components of the method (paragraphs listed above and claim 2, *supra*). Fijikawa simply has no bearing whatsoever on the current invention.

The examiner attempts to instruct appellant on what is required of a written description:

> An applicant may also show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics which provide evidence that applicant was in possession of the claimed invention, i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics. (OA1, Exh. "D", OA2, Exh. "E").

The examiner then argues that:

> applicant's disclosure of chemical species, an amino group, a amine, a drug, a first substance, and a second substance" does not adequately describe the claimed genera, the examiner stating "The mere fact that Applicant may have discovered one type of chemical species may be loaded into the lipid-like vesicles and achieve the desire (sic) stability is not sufficient to claim the entire genus. OA1, OA2.

It should first be noted that the examiner has short changed appellant's invention and disclosure thereof by only mentioning basic entities specifically. Appellant also specifically claims acidic species. Both acidic and basic species are well-described in the specification. For example, on page 3, paragraph [0023], it is taught that:

> The term chemical species having one or more selected acid or basic pH responsive groups is also used broadly to indicate any chemical or drug having acid or basic groups, properties or functions such as, but not limited to amine or carboxyl groups. ... The term also includes any chemical that has desired chemical or therapeutic properties that will not be sufficiently altered by the attachment of such pH responsive groups. ... Examples of drugs having ... basic properties are viscrisine, doxorubicin, streptomycin, chloroquine and daunorubicin. Examples of drugs ... having acidic properties are derivatives of methotrexate, daunomycin, penicillin, p-amino salicylic acid and salicylic acid derivatives. '922 application, Exh. "F", paragraph [0023].

What could be more clear? Any chemical species having, or that can be derivatized to have without altering its desirable properties, a basic or acid substituent group, is within the scope of the claimed invention. Appellant believes that it would be fair to take judicial notice of the fact that anyone who has successfully completed a fundamental university course in organic chemistry knows what substituent groups impart basic and what substituent groups impart acidic properties on a compound. Further, it might also be noticed that the skill level of those in the relevant art is very likely substantially higher than the preceding. For the purposes of this invention, the use of any such compound is encompassed: The claimed method as conceived and practiced by appellant is intended to cover such and its scope is in fact intended to be that pervasive.

Finally, the examiner states that:

> The written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice ... or by disclosure of relevant indentifying characteristics coupled with a known or disclosed correlation between function and structure ... Eli Lilly, 119F3d at 1568...
>
> A "representative number of species" means that the species which are adequately described are representative of the entire species. Thus, when there is substantial variation within the genus, one must describe a sufficient variety of species to reflect the variation within the genus. The disclosure of only one species encompassed with a genus adequately describes a claim directed to that genus only if the disclosure "indicates that the patentee has invented species sufficient to constitute the genus." OA1, OA2, emphasis added.

It is first noted that appellant has not "invented species." Appellant has invented a method of incorporating a broad spectrum of compounds into lipid-like particles and keeping them there for relatively lengthy periods of time. In addition, there are only two species: those with acid responsive groups and those with base responsive groups.

While the examiner is correct in noting that the written description of a claimed species **may** be satisfied by sufficient examples, the statement is pregnant with other possbilities; i.e., it may not require any particular number of examples and in fact may not require examples at all. That is, the number of required exemplary species, if any, is determined on a case-by-case basis. In fact the Federal Circuit very recently stated, with regard to a patent challenged in part because its specification contained **no** working examples:

> ...a patent claim is not necessarily invalid for lack of written description just because it is broader than the specific examples disclosed. Martek Biosciences v. Nutrinova, 579 F.3d 1363 (2009)

and then the court opined, with regard to the testimony of an expert witness:

> He relied on specific statements in the 1988 application and explained how, in his opinion, a person of ordinary skill in the art would understand those statements. Id.

The current specification is rife with discussion and description of what constitutes acid or base responsive groups ('922 application, Exh. "F", paragraph [0023], , what the relationship of the pHs of the various constituents of the method must be in order for the method to work ('922 application, Exh. "F", paragraphs [0023] – [0034]) and, further, provides specific examples of the incorporation of such acid or base species into lipid-like vesicles ('922 application, Exh. "F", Examples 1, 3, 4 and 5) and even provides *in vivo* data to demonstrate that the method as claimed is well-tolerated by animals ('922 application, Exh. "F", Example 2). Those of ordinary skill in the art would have no difficulty at all understanding the current invention and realizing that appellant without question had possession of the entire claimed scope at the time the application was filed.

For the sake of brevity, the Board is respectfully referred to Appellant's responses to OA1 and OA2, which responses are attached hereto as Exhibits "G" and "H" of the Evidence Appendix, for additional comments as to how and why the current specification unquestionably meets the written description requirement of 35 U.S.C. § 112, first paragraph.

Claims 1-12 are patentable over Nichols, et al., Deamer, et al. or Cramer; i.e., none of these references renders the claimed invention obvious

With regard to Nichols, the examiner's position is that it does teach the loading of an amine into a liposome using a pH gradient. This appellant does not dispute. The crux of the current invention, as was pointed out to the examiner on several occasions, is that the concentration of the loaded amine – or of any basic or acidic chemical substance – can be maintained in the lipid-like vesicle, i.e. liposome, for a substantial period of time after the pH gradient has been destroyed. This is an unexpected, surprising, utterly novel development that in no way could have been gleaned from Nichols by those skilled in the art. In fact, Nichols says nothing whatsoever about what happens to the loaded material after loading.

As for Deamer, the examiner argues the it teaches a method of preparation of liposomes using the method of claim 1; that is, that Deamer loads amines by preparing liposomes with acid pH and titrating them with a base to create a pH gradient and

adding a basic amine. Again, as was the case with Nichols, at no point does Deamer discuss, allude to suggest or so much as tangentially hint at what happens when the pH gradient is destroyed and a membrane impermeable buffer has been used within the lipid-like vesicle. This is at the very core of the present invention and it demonstrates how kinetics can trump thermodynamics if the system is carefully designed as set forth in the current invention.

Finally, with regard to Cramer the situation is exactly the same: Cramer is concerned solely with the phenomenon of pH-induced transport of carboxylic acids across a liposomal membrane. The only reference to elution of the incorporated species from the liposomes is the statement that "[T]he nonselective leakage of both fumaric and maleic acids depicted in Figure 4 at longer times is probably the result of vesicle rupture in response to the osmotic stress provided by the early selective transport of protonated fumaric acid." It is important to note that <u>this phenomenon occurs while the pH gradient is still intact</u>, which is diametrically opposite what occurs in the current invention. There is no mention of using membrane impermeable buffers or of what happens once the pH gradient is destroyed as is the case in the current method.

Supplemental comments

In addition to the arguments above addressing the examiner's specific arguments against patentability of the current invention, appellant offers the following comments, which appellant respectfully requests be considered:

The major new elements of the current invention, none of which could be readily discerned from the prior art by those of ordinary skill in the art, are:

(1) that drugs or other chemical species upon loading into lipid-like vesicles such as liposomes could be retained within the vesicle upon dilution into a large external volume as would be the case if they were injected into the body of an animal. The prior art merely suggested that there would be a concentration gradient across the liposome membrane corresponding to the driving force of electrochemical gradients, which does not in any manner relate to whether much larger concentration gradients could be maintained due to the <u>kinetics</u> of the release of the drug or other chemical species from the liposomes after dilution into a large external volume (q.v., discussion of the lacking elements in Nichols, Deamer and Cramer, *supra*). That is, under the proper conditions

as set forth in the current invention, kinetics play a prominent role in retention of a drug or other chemical species in a lipid-like vesicle as epitomized by liposomes;

(2) that even very simple molecules like carboxylic acids (derivatized Tempamine, '922 application, Exh. "F", Example 3) and amines (Tempamine itself, '922 application, Exh. "F", Example 1) can be loaded into a lipid-like vesicle using the claimed inventive method and the concentration thereof in the vesicle can be maintained for a substantial period of time, whereas the prior art clearly teaches that the movement of such simple molecules across a liposomal membrane occurs very rapidly. In fact, the literature in general indicates that weak acids and bases can be used to study rapid trans-membrane ion movement occurring on a sub-second time scale in clear opposition to the situation in the present invention; and

(3) that it would not generally be expected by those skilled in the art that liposomes having large electrochemical gradients across their membranes would be well-tolerated by animals upon injection. Rather, conventional wisdom at the time held that it would likely be that the physiological effects of endogenous amines, which control numerous physiological processes and whose tissue distribution could well be altered by such electrochemically charged liposomes, would be compromised. To the contrary, the current invention demonstrates (Example 2 and Example 5) that the inventive drug-loaded liposomes are well-tolerated in mice.

CONCLUSION

The examiner has failed, as a matter of law, to show that the current invention lacks an adequate description and/or that it is obvious over Nichols, Deamer or Cramer. Appellant therefore respectfully requests that the Board reverse the examiner's rejection and order that the application proceed to issue.

Date: 30 November 2009

Respectfully submitted,

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CLAIMS APPENDIX

The claims on appeal are:

1. A method of loading lipid-like vesicles, comprising:

forming lipid-like vesicles in a solution comprising an acidic buffer if the chemical species to be loaded is basic or a basic buffer if the chemical species to be loaded is acidic; wherein:

membranes of the formed lipid-like vesicles are impermeable to the buffer;

adjusting the pH of the solution exterior to the membranes of the lipid-like vesicles to a basic pH if the chemical species to be loaded is basic or to an acidic pH if the chemical species to be loaded is acidic;

adding a basic chemical species to the adjusted basic exterior solution or an acidic chemical species to the adjusted acidic exterior solution;

loading the chemical species into the vesicle; and

adjusting the exterior solution to a physiologically benign pH; wherein:

the chemical species is substantially maintained in the vesicle for at least one quarter hour after the adjustment of the exterior solution.

2. A method of loading lipid-like vesicles having a membrane permeable to a chemical species to be loaded and for substantially maintaining the loaded chemical species within the vesicle for at least one-quarter hour following loading by inducing a pH gradient across the membrane, comprising:

(1) incorporating within the vesicle a buffer solution buffered to a selected acid or alkaline pH having a selected molarity and at least one selected pKa approximately equal to the selected buffer pH, the membrane being substantially impermeable to the buffer for at least one-quarter hour following loading of the chemical species;

(2) positioning the vesicles in a bulk solution having a selected pH; and

(3) providing the bulk solution with a chemical species having one or more selected acid pH responsive groups if the buffer is alkaline or one or more basic pH responsive groups if the buffer is acidic wherein the pH of the bulk solution is

at least 0.5, 0.3 or 0.2 of a pH unit higher than the pH of the buffer if the buffer is acidic and the chemical species has one, two, or three or more basic pH responsive groups, or the pH of the bulk solution is at least 0.5, 0.3 or 0.2 of a pH unit lower than the pH of the buffer if the buffer is basic and the chemical species has one, two or three or more acid pH responsive groups, the pH responsive groups of the chemical species having one or more acid pH responsive groups have a pKa that is generally lower than or equal to the pH of the bulk solution and generally higher than or equal to 3.5 and the pH responsive groups of the chemical species having one or more basic pH responsive groups have a pKa that is generally higher than or equal to the pH of the bulk solution and generally lower than or equal to 11.

3. A method according to claim 2 wherein the pH responsive group or groups are acid pH responsive groups and the buffer has a pKa of about 10.

4. A method according to claim 3 wherein the chemical species has a pKa of about 4-7.

5. A method according to claim 4 wherein the pH responsive group is a carboxyl group.

6. A method according to claim 2 wherein the pH responsive group or groups are basic pH responsive groups, and the buffer has a pKa in the range of about 5.

7. A method according to claim 6 wherein the chemical species has a pKa from about 7-10.

8. A method according to claim 7 wherein the pH responsive group is an amino group.

9. A method according to claim 8 wherein the chemical species is an amine.

10. A method according to claim 2 wherein the vesicle is prepared in the buffer and incorporates the buffer via mixing and sonication.

11. A method according to claim 2 wherein the pH of the bulk solution is about 7.0 to about 7.8.

12. A method according to claim 11 wherein the pH of the bulk solution is about 7.4.

17. A kit as set forth in claim 16 wherein said chemical species is a drug.

18. A kit as set forth in claim 17 wherein the mixture will have a pH that is physiologically benign in regard to the blood of a mammal.

19. A kit as set forth in claim 18 further comprising means for parenterally delivering the mixture to a mammal in vivo.

20. A kit for loading lipid-like vesicles having a membrane permeable to an acid or basic chemical species to be loaded comprising:

(1) a first compartment having a first solution having membranous lipid-like vesicles incorporating a buffer buffered to a selected basic pH if the chemical species to be loaded is an acid or acid pH if the species in a base, the buffer having a selected pKa and a selected molarity, the membrane being substantially impermeable to the buffer for at least one-quarter hour following loading of the chemical species, the first solution having a selected pH such that the stability of the vesicle and its buffer will be maintained for a period of at least one week at 4 °C;

(2) a second separate compartment having a first substance which when combined with the first solution will adjust the pH of the first solution so as to provide a predetermined pH gradient between the buffer within the vesicle and the pH adjusted first solution; and

(3) a third separate compartment having a second substance which when combined with the pH adjusted first solution will further change the pH of said solution to a physiologically benign value with regard to the blood of a mammal.

21. A kit as set forth in claim 20 further comprising a selected chemical species.

22. A kit as set forth in claim 21 wherein the selected chemical species is a drug.

23. A kit as set forth in claim 22 further including a means for parentally delivering the vesicle solution having the physiologically benign adjusted pH to a mammal in vivo.

24. A method of detoxifying an animal suffering from an overdose of a chemical species with basic pH responsive groups comprising injecting the animal with a solution having a physiologically benign pH with respect to the animal, the solution having large volumes of liposomes having a buffer solution buffered to a pH generally lower than or equal to 5.4 and the buffer having at least one selected pKa and a selected molarity within the physiological range of the animal the liposomes being substantially impermeable to the buffer for at least one hour after injection.

EVIDENCE APPENDIX

Attached hereto are the following Exhibits:

A. Nichols, et al., Catecholamine Uptake and Concentration by Liposomes Maintaining pH Gradients, Biochimica et Biophysica Acta, 1976, 455:269-271

B. Deamer, et al., The Response of Fluorescent Amines to pH Gradients Across Liposome Membranes, Biochimica et Biophysical Acta, 1972, 274:323-335

C. Cramer, et al., NMR Studies of pH-Induced Transport of Carboxylic Acids Across Phospholipid Vesicle Membranes, Biochemical and Biophysical Research Communications, 1977, 75:295-301.

D. Office Action mailed 5 September 2008

E. Office Action mailed 31 March 2009

F. Published Patent Application Serial No. 2004/0208922, filed 20 January 2004

G. Response to Office Action mailed 29 November 2007, filed 29 May 2008

H. Response to Office Action mailed 5 September 2008, filed 5 January 2009

EXHIBIT A

Biochimica et Biophysica Acta, 455 (1976) 269—271

BBA Report

BBA 71276

CATECHOLAMINE UPTAKE AND CONCENTRATION BY LIPOSOMES MAINTAINING pH GRADIENTS

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(Received August 20th, 1976)

Summary

Liposomes were prepared with pH gradients across their membranes (acidic interiors with respect to the external buffer). These liposomes efficiently concentrated several catecholamines (dopamine, norepinephrine, and epinephrine) added to the external buffer. Our observations support a mechanism which suggests that pH gradients may contribute to uptake of catecholamines by sub-cellular storage sites.

A pH gradient can profoundly influence the distribution of weak acids and bases across membranes. In past studies, this effect has been used to measure pH gradients in mitochondria [1] with a weak acid, and in chloroplasts [2], lysosomes [3], chromaffin granules [4] and liposomes [5] with amines such as methylamine and 9-aminoacridine. In the present study, using liposomes as a model system, we have tested the possibility that catecholamines may be concentrated by pH gradients.

Ideally, the charged form of the amine is assumed to be unable to penetrate a membrane while the uncharged form can freely penetrate. For this reason, equal concentrations of the uncharged form of the amine will exist across a membrane at equilibrium, while the charged form will be distributed in proportion to the difference in pH. Net distribution of the amine is described by the relationship

$$\frac{[A^T]_i}{[A^T]_o} = \frac{K_a + [H^+]_i}{K_a + [H^+]_o}$$

where $[A^T]_i$ and $[A^T]_o$ are the total internal and external concentrations of the amine, $[H^+]_i$ and $[H^+]_o$ are the internal and external proton concentrations, and K_a is the dissociation constant of the amine [2, 5]. It follows that a

pH gradient, acid inside, will drive accumulation of amines into the internal volume. We will show here that the distribution of catecholamines across liposome membranes maintaining a pH gradient is qualitatively similar to that which would be expected from the above equation.

Liposomes were prepared by injecting 7 ml of egg phosphatidylcholine dissolved in ether (2 μmol/ml) into 14 ml of citrate-phosphate buffer, pH 5.0 at 55°C. When injected under these conditions, the ether vaporizes and the phospholipid forms 0.05—0.2 μm diameter vesicles which efficiently trap buffer [6]. Liposome-buffer mixtures were filtered through a 1.2 μm millipore filter to remove a small fraction of larger, multilamellar liposomes. The liposome-buffer mixtures were titrated with NaOH to pH 8, establishing an initial gradient of 3 pH units. Tritium-labelled catecholamine (epinephrine, norepinephrine, dopamine) was then added to the liposome-buffer mixture to a final concentration of 10 μM (5000 cpm/0.1 ml). 2-ml aliquots of the liposome-buffer incubation mixture were withdrawn at timed intervals and filtered through a Sephadex G-50 column using a pH 8 citrate-phosphate buffer for elution. This procedure separated the liposomes containing catechol-

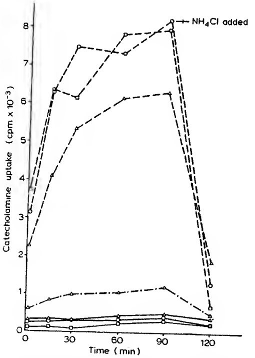

Fig. 1. Accumulation of catecholamines by liposomes maintaining three different pH gradients. Liposomes were prepared in citrate-phosphate buffer (pH 5.0). Liposome-buffer mixtures were titrated with NaOH to pH 8.0, establishing an initial gradient of 3 pH units. Tritium-labelled catecholamine was then added to the liposome-buffer mixture. Aliquots of the liposome-buffer incubation mixture were withdrawn at timed intervals. The liposomes were separated from the incubation buffer by filtering through a Sephadex G-50 column and counted. After 90 min, NH_4Cl was added to the remaining incubation mixture and a last sample was filtered at 120 min. — — —, pH gradient of 3 units; — · —, pH gradient of 1.5 units; ——, pH gradient of 0. △, [^{3}H]norepinephrine; □, [^{3}H]dopamine; ○, [^{3}H]epinephrine. Under similar conditions, no uptake of [^{14}C]acetylcholine could be detected.

amines from free catecholamines. After 90 min, 0.1 ml of 1 M NH_4Cl was added to the remaining incubation mixture and a last sample was filtered at 120 min. Addition of NH_4Cl destroys the pH gradient across the liposome membranes [5], permitting us to test for the pH dependence of any catecholamine accumulation. The tritium-labelled catecholamine trapped inside the liposome was counted on a Beckman CPM-100 liquid scintillation counter. Estimates of the degree of concentration were made by comparing the uptake in the presence of a pH gradient with that of controls in the absence of a pH gradient.

Under these conditions, we observed a remarkable accumulation of each of the catecholamines tested. Uptake into the liposomes occurred relatively slowly, and was maximal 90 min after addition of the amine. At this time we found concentrations of 12-fold over controls for norepinephrine. Epinephrine and dopamine were concentrated 18- and 23-fold respectively. In earlier experiments with Dr. Enrique Ochoa in 1975 (unpublished) we found that the indolamine serotonin was concentrated 40-fold over controls. When the gradients were destroyed by ammonium chloride additions, the accumulated catecholamines were released, demonstrating that the uptake was reversible and dependent upon pH gradients, Finally, when [^{14}C]acetylcholine was tested under similar conditions, no uptake was measureable.

It should be noted that the observed accumulation, 10—20-fold over controls, is only a fraction of that expected for an ideal monoamine responding to a 3 pH unit gradient. This is probably due to decay of the pH gradient over time. We have measured the decay rate in this system using 9-aminoacridine as a Δ pH indicator [5, 6] and found that half the pH gradient was lost in 30 min, in agreement with earlier results [6].

It is also interesting that the uptake of the catecholamines is relatively slow. For instance, the monoamine 9-aminoacridine reaches equilibrium within seconds under similar conditions [5]. The slow uptake is probably a function of the hydroxyl groups on the catecholamines, which would limit their permeability to lipid bilayer membranes.

These results suggest a potential role of pH gradients in the uptake and concentration of catecholamines by sub-cellular storage sites, as was suggested earlier by Johnson and Scarpa [4] for chromaffin granules. Our results are also consistent with Johnson and Scarpa's observation that chromaffin granules maintain acidic interiors (pH 5.5) with respect to a more alkaline external medium.

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EXHIBIT B

BIOCHIMICA ET BIOPHYSICA ACTA

BBA 75984

THE RESPONSE OF FLUORESCENT AMINES TO pH GRADIENTS ACROSS LIPOSOME MEMBRANES

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SUMMARY

Phospholipid liposomes were used to test atebrin and 9-aminoacridine as fluorescent probes for measuring pH gradients across membranes. Quenching of 9-aminoacridine fluorescence could be quantitatively related to the magnitude of pH gradients across liposome membranes, and the relation indicated that the distribution of this amine between inner and outer volumes in the liposome system was that of an ideal monoamine. Quenching of atebrin fluorescence could not be related to that predicted from the theoretical equation of a diamine. We conclude that 9-aminoacridine is the preferred fluorescent probe for use in the range of ΔpH = 2–4 pH units.

Quenching of 9-aminoacridine fluorescence was used to measure the development of pH gradients in liposomes. In one system, an oxidation–reduction reaction mediated by a dye which accepted H^+ upon reduction, was established across liposome membranes. It was found that gradients of at least 4 pH units could develop under these conditions. In a second system, nigericin mediated the exchange of K^+ for H^+ across the liposome membranes, and it was found that the pH gradient developed depended upon the original K^+ gradient and could be at least 2.2 pH units. Liposomes offer a model membrane system in which controlled pH gradients may be established.

INTRODUCTION

Proton transport and resulting gradients of H^+ activity across membranes are now recognized to be integrally related to phosphorylation and ion transport in chloroplasts, mitochondria and other membranous systems. Present methods for estimating pH gradients across membranes involve measurements of pH changes in the external medium using a glass electrode or indicator dyes[1,2], the distribution of amines[3] or weak acids[4] in response to pH gradients, or other pH-related absorption or scattering changes[5,6]. Each of these methods has certain limitations, and a technique which more directly and rapidly measures pH gradients would be useful.

Kraayenhof[7] found that fluorescence of the diamine, atebrin, was quenched

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Abbreviations: MES, 2-(*N*-morpholino)ethanesulphonic acid; TES, *N*-tris(hydroxymethyl)-methyl-2-aminoethane sulphonic acid; PMS, phenazine methosulphate.

during electron transport in chloroplast suspensions, and suggested that the quenching may be related to the high energy state of the chloroplasts. Rottenberg *et al.*[3] extended this work with atebrin and 9-aminoacridine, and proposed that the quenching may depend on inward movement of the amines in response to pH gradients which develop across chloroplast membranes during electron transport. Rottenberg *et al.* noted that fluorescence quenching may therefore be a parameter of amine uptake and calculated from the amine distribution that a pH gradient as large as 3.5 pH units could develop across the membranes of illuminated chloroplasts.

This potentially important method has two limitations. It is not known if fluorescence is completely quenched by entry into membrane enclosed volumes, and the assumption that the amines respond ideally to pH gradients across membranes is open to question. To test these points, we have studied the quenching of atebrin and 9-aminoacridine fluorescence in phospholipid liposomes[8], in which controlled pH gradients were established. In a second series of experiments, the fluorescent probes were used to determine whether pH gradients may be generated across liposome membranes by a simple electron transport system or an ion exchange mediated by the antibiotic nigericin.

METHODS

Lipids

Egg lecithin was prepared from hens eggs by column chromatography on alumina and silicic acid. The product was chromatographically pure as judged by thin-layer chromatography on Silica Gel G. Dicetyl phosphate was a kind gift of Dr A. Bangham. Bacteriochlorophyll was prepared from a methanol extract of *Rhodopseudomonas capsulata* by column chromatography on Whatman cellulose powder using 5% diethyl ether in light petroleum.

Fluorescent probes

Atebrin was purchased from Sigma, and 9-aminoacridine from Ralph N. Emanuel Ltd, Wembley, England. Both were dissolved in distilled water at a concentration of 1 mM.

Buffers

It was found that organic buffers (Tricine, 2-(*N*-morpholino)ethanesulphonic acid (MES), *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES)) quenched 9-aminoacridine fluorescence to variable degrees, although atebrin fluorescence was unaffected. To avoid the partial quenching, a buffer containing 0.1 M sodium phosphate and 0.1 M sodium pyrophosphate was used for the pH range 5.0 to 9.0. Since liposome membranes are relatively permeable to chloride[8,9] it was possible that any chloride in the medium could move with a proton and discharge pH gradients. Therefore, buffer pH was adjusted with H_2SO_4, rather than HCl. The pH gradients are also sensitive to amines and care was taken that no amine compounds other than the fluorescent probes were inadvertently introduced into the medium. When K^+- and Na^+-free media were required, phosphoric acid was neutralized with tetramethylammonium hydroxide.

Liposome preparations

Liposomes were produced by evaporating chloroform solutions of varying amounts of egg lecithin *plus* 5 % dicetyl phosphate under nitrogen. The lipid was sonicated for 1 min with 2 ml of the medium to be trapped inside the liposome volume. A Soniprobe 1130A (Dawe Instruments, London) was used in this step at a power setting of 50 W. During sonication, the temperature of the solution rose to approximately 40 °C and this presumably aided formation of liposome vesicles[10]. In some experiments, sonication time was varied from 0 to 30 min and the amount of trapped anion (phosphate or ferricyanide) was measured. It was found that the amount of trapped material was maximal after 30–60 s sonication. In view of the report that prolonged sonication causes some degradation of phospholipid[11] 1 min seemed a reasonable time interval. The solutions to be trapped contained either phosphate–pyrophosphate buffer at pH 5.0 to 9.0, 0.1 M $K_3Fe(CN)_6$ or varying concentrations of K_2SO_4. All solutions included 1 mM EDTA. The sonicated suspension (1.5 ml) was placed on a 15 cm × 1 cm column of Sephadex G-50, course grade[9], which was equilibrated with sucrose solution containing 10 mM buffer at the original pH. Sucrose was added to osmotically balance the vesicles during gel filtration. For instance, 0.1 M $K_3Fe(CN)_6$ would be balanced with 0.4 M sucrose. The turbid liposome suspension was collected from the column in 3–4 ml of the eluate and adjusted to a known volume (3–5 ml) with sucrose solution. Examination of the preparation by phase-contrast microscopy showed a mixed population of liposome. The major species were 0.1–1 μm vesicles apparently with single membranes, but with occasional larger multilayered liposomes. In some experiments, 2 mole % bacteriochlorophyll was included in the lipid prior to evaporation and sonication. All experiments were carried out within 6 h of sonication.

Fluorescence measurements

Fluorescence of the amines was measured in a fluorimeter with the photomultiplier at 90° to the actinic light path. The actinic beam was provided by a quartz–iodine bulb (Philips 12258/99 12 V 55 W) supplied by a stabilized power supply (Coutant Electronics Ltd, Reading, England). Blue light was selected either by a monochromator (Hilger and Watt Ltd, London, England Type D 292) or by a Wratten 36 gelatin filter (Kodak Ltd, London) together with a Corning 9782 glass filter. The photomultiplier (EMI Electronics Ltd, Hayes, Middlesex, England, Type 9601 B) was screened by a Wratten 61 filter, together with a Corning 9782 glass filter. The output was fed to a recorder (Toa Polyrecorder EPR-2TB, T.E.M. Sales Ltd, Crawley, Sussex, England) by way of a simple amplifier and backing circuit.

RESULTS

Effect of pH on intrinsic fluorescence

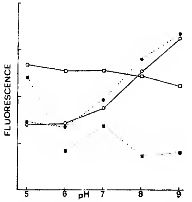
As noted in previous studies[7,12] atebrin fluorescence is pH dependent, and increased nearly 2-fold as pH increased from 6 to 9 (Fig. 1). This is related to the first pK of this diamine (pK = 7.9, 10.5) and the fluorescence change is half maximal at pH 7.9. There was little variation in 9-aminoacridine fluorescence (pK = 10.0) in inorganic buffers over the same pH range. It is important to note the marked quen-

ching effect of organic buffers on 9-aminoacridine fluorescence (Fig. 1). Atebrin fluorescence was not significantly affected by organic buffers.

Fluorescence quenching and enhancement dependent on pH gradients

Fig. 2 shows a typical experiment in which liposomes were added to media containing atebrin or 9-aminoacridine. When the liposomes contained buffers at pH lower than that of the medium, atebrin fluorescence was enhanced, rather than quenched (Fig. 2A). At higher lipid concentrations the enhancement may be 250 %. Triton (0.1 mM) or NH_4Cl (5 mM) completely reversed the enhancement. However, if 2 mole % bacteriochlorophyll was included in the lipid phase, quenching of fluorescence occurred under the same conditions, and again the quenching was reversible with Triton or NH_4Cl (Fig. 2B). Quenching of 9-aminoacridine fluorescence occurred both with and without bacteriochlorophyll, although there was somewhat increased quenching when bacteriochlorophyll was present (Figs 2C and 2D).

The effect of a range of pH gradients on atebrin and 9-aminoacridine fluorescence quenching by liposomes is shown in Fig. 3. In both cases the inner phase was maintained at pH 5 and the external pH was varied from 5 to 9. It is apparent that quenching of the fluorescence of the amines in liposome suspensions was strongly dependent on pH gradients across the liposome membrane. In the absence of bacteriochlorophyll the enhancement of atebrin fluorescence was maximal at pH 6.2 (ΔpH = 1.2), then decreased. If bacteriochlorophyll was present, the quenching curve of



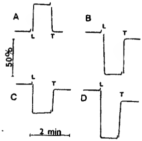


Fig. 1. Dependence of amine fluorescence on pH. The fluorescence (arbitrary units) of atebrin and 9-aminoacridine was measured in organic buffers. The organic buffers (-----) were 0.1 M MES (pH 5 and 6), 0.1 M TES (pH 7) and 0.1 M Tricine (pH 8 and 9). The inorganic buffer (—) was the phosphate–pyrophosphate buffer described in Methods. ■ and □, 4 μM 9-aminoacridine in organic and inorganic buffers, respectively; ● and ○, 4 μM atebrin in organic and inorganic buffers, respectively.

Fig. 2. Amine fluorescence changes in liposome suspensions. Liposomes (L) containing buffer at pH 5.0 were added to solutions of atebrin and 9-aminoacridine, followed by Triton X-100 (T) to a final concentration of 0.1 mM. Final lipid concentration was 0.08 mg/ml. (A) Fluorescence enhancement occurred when liposomes were added to 4 μM atebrin in inorganic buffer (pH 7.0). No bacteriochlorophyll was present. (B) Fluorescence quenching occurred under conditions for (A) but with 2 mole % bacteriochlorophyll in the liposome membranes. (C) Fluorescence quenching occurred when liposomes were added to 4 μM 9-aminoacridine in inorganic buffer (pH 8.0). No bacteriochlorophyll was present. (D) Increased fluorescence quenching occurred under conditions for (C) but with 2 mole % bacteriochlorophyll in the liposome membranes.

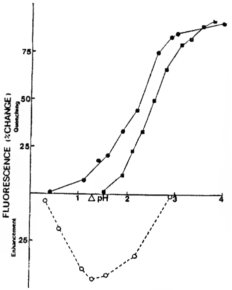
atebrin fluorescence was similar to that of 9-aminoacridine fluorescence, but as noted previously[3] atebrin was more responsive to pH gradients.

For monoamines it is possible to derive the relation

$$\log \frac{[H^+]_i}{[H^+]_o} = -\Delta\, pH_{(i-o)} = \log \frac{A_i}{A_o} + \log \frac{V_o}{V_i}$$

where V_o/V_i is the ratio of external volume to internal volume and A is the total amount of amine cation in the inner or outer volumes. Thus, $-\Delta$pH plotted against log A_i/A_o should provide a linear relation of slope = 1.0 with an intercept equal to log V_o/V_i. When pK_a for the amine is much higher than the experimental pH, the concentration of free amine is negligible and A approximates closely to the total amine. We have treated data from the experiments using 9-aminoacridine in this form, assuming that A_i/A_o is equivalent to $Q/(100-Q)$ where fluorescence in the absence of a pH gradient is taken as 100 and Q is the measured percent quenching. Furthermore, we varied the volume ratios since the intercept equal to the log of the volume ratios, should also vary in a predictable manner. These results are shown in Fig. 4.

In this experiment, five different liposome concentrations were used so that V_o/V_i varied in a controlled manner, and ΔpH was varied from 2 to 4 pH units. Since we do not have a precise measurement of liposome volume, we assumed that the results



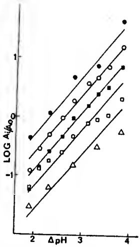


Fig. 3. Effect of varying pH gradients on fluorescence changes. ○---○, enhancement of atebrin fluorescence in presence of liposomes lacking pigment; ●—●, quenching of atebrin fluorescence by liposome containing 2 mole % bacteriochlorophyll; ■—■, quenching of 9-aminoacridine fluorescence by liposomes containing 2 mole % bacteriochlorophyll. Liposomes contained buffer at pH 5.0. Lipid concentration, 0.08 mg/ml; amine concentration, 4 μM.

Fig. 4. Effect on the distribution of 9-aminoacridine, as estimated from the quenching of fluorescence, of varying lipid concentration, and pH gradients across liposome membranes. Liposomes containing buffer (pH 5.0) were added to 2.5 ml of buffer ranging from pH 7 to 9. Bacteriochlorophyll, 2 mole %, was included in the lipid. Lipid concentrations: ●—●, 0.24 mg/ml; ○—○, 0.12 mg/ml; ■—■, 0.06 mg/ml; □—□, 0.03 mg/ml; △—△, 0.012 mg/ml.

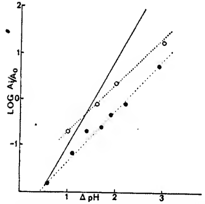
for a liposome concentration of 0.12 mg/ml were correct. Theoretical lines with a slope of 1.0 and intercepts corresponding to the relative volume ratios were then drawn through all five sets of data. The fit of the data to the theoretical lines was quite good for the three higher liposome concentrations, but deviated to some extent from the expected lines for the lower concentrations.

A similar experiment was carried out for two concentrations of liposomes in which atebrin was the fluorescent amine. Atebrin did not behave as an ideal diamine (Fig. 5) since the results deviated considerably from the slope of the theoretical line, which was drawn by graphing the logarithmic form of the distribution equation for an ideal diamine using the values of pK_a for atebrin.

Developing pH gradients across liposome membranes

There are a number of methods by which one might expect to develop pH gradients across liposomes. Two of the most convenient are described here. In the first, an oxidation–reduction reaction was established across the liposomal membrane. This type of system has been described earlier by Hinkle[13] and Kimelberg *et al.*[14]. In the present system liposomes containing an oxidant were added to a medium containing a reductant. A lipid-soluble carrier molecule which acted as a hydrogen acceptor upon reduction was then added. The carrier would be expected to transport both protons and electrons inward and release protons inside following oxidation by the trapped couple.

A second method involved the use of the ionophoric antibiotic, nigericin, which



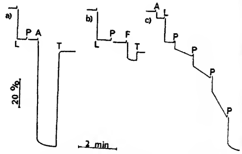


Fig. 5. Effect of varying pH gradients and lipid concentration on apparent distribution of atebrin across liposome membranes. Same conditions as Fig. 4, but buffers ranged from pH 6 to 8 and 4 μM atebrin was used. Lipid concentrations: ●---●, 0.12 mg/ml; ○---○, 0.3 mg/ml. Solid line is a theoretical line for the equation of diamine distribution assuming V_o/V_i = 1000. See text for details.

Fig. 6. Quenching of 9-aminoacridine fluorescence by liposomes containing $K_3Fe(CN)_6$. Liposomes (L) were added to 2.5 ml of buffer solution at pH 7.0, followed by additions of PMS (P), 2 mM ascorbic acid (A) or $K_4Fe(CN)_6$ (F), and 0.1 mM Triton X-100 (T). Lipid concentration was 0.08 mg/ml and contained 2 mole % bacteriochlorophyll. (a) If 4 μM PMS was present initially addition of ascorbic acid caused a nearly complete quenching of fluorescence which was reversible by Triton. (b) Addition of ferrocyanide under the conditions of (a) produced a much smaller quenching. (c) Addition of small increments of PMS (each addition was an increase of 0.4 μM) in the presence of ascorbic acid produced an initial rapid quenching phase, followed by a slower phase. As total PMS concentration was increased, the rate of the slower phase also increased.

mediates the exchange of K^+ and H^+ across liposome membranes[15]. If nigericin is added to a membrane system across which a K^+ gradient exists, K^+ exchanges with protons across the membranes and a pH gradient should develop. We have tested both of the above methods in the liposome system, using 9-aminoacridine to detect and measure pH gradients.

In the redox couple method ferricyanide was used as an oxidant trapped inside the liposomes, ascorbic acid or ferrocyanide was used as a reductant outside and phenazine methosulfate as a proton carrier molecule. A concentration of 0.1 M $K_3Fe(CN)_6$ was used and approximately 0.39 µmole/mg lipid could be trapped in the liposome volume. In a typical experiment (Fig. 6A) no quenching was observed until both ascorbate and phenazine methosulphate (PMS) were added, at which time a large (>90%) quenching occurred which was completely reversed upon addition of Triton or NH_4Cl. Ferrocyanide was not as effective in producing a large quenching (Fig. 6B). If the data in Fig. 4 were used to calibrate ΔpH for the lipid concentration present in the ferricyanide system, it would indicate that a ΔpH of 4.0 units developed across the liposome membranes when ascorbate was used as the reductant, and 1.6 units when ferrocyanide was used as a reductant.

The mechanism by which PMS mediated the redox reaction was of some interest. PMS could either act in a cyclic manner, in which case catalytic amounts would promote the electron transport reaction, or it could act in a non-cyclic manner, in which case the extent of the reaction would be limited by the amount of PMS present. This was tested by titrating the quenching with small additions of PMS (Fig. 6 C). It is clear that the quenching which occurs after PMS addition has both a fast and slow phase. The significance of this result will be discussed later.

Results from the second method for developing pH gradients are shown in Fig. 7. When liposomes were loaded with K_2SO_4 and placed in a K^+-free medium, addition of nigericin induced a rapid quenching of 9-aminoacridine fluorescence (not shown) similar to that seen with the redox couple system. If it is assumed that nigericin mediates the neutral exchange of one K^+ per H^+, then after addition of

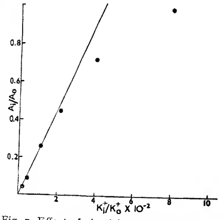


Fig. 7. Effect of nigericin and K^+ gradients on apparent 9-aminoacridine distribution. Liposomes were loaded with 0.1 M K_2SO_4 and placed in 2.5 ml solutions containing varying concentrations of K^+. Nigericin (1 µg) was then added, and A_i/A_o was calculated from the resulting quenching. The lipid (concentration, 0.08 mg/ml) contained 2 mole % bacteriochlorophyll. Solid line: theoretical curve (see text).

nigericin $A_i^+/A_0^+ = H_i^+/H_0^+ = K_i^+/K_0^+$. To test this relation, liposomes containing 0.1 M K_2SO_4 were suspended in varying concentrations of K^+ and A_i/A_0 was plotted against initial K_i^+/K_0^+. The experimental data fit the theoretical line at values of $K_i^+/K_0^+ \simeq 200$. Above this value the amount of K_i^+ which was lost to the medium in exchange for protons changed the value of K_0^+ so that the equilibrium values differed significantly from the initial values plotted and the data deviate from the theoretical line.

DISCUSSION

Fluorescent probes which respond to pH gradients across membranes may be either amines or weak acids, although none of the latter have been tested as yet. Ideally, fluorescent probes would have the following properties:

(1) They would be monoamines or monofunctional weak acids to simplify theoretical treatment.

(2) Fluorescent quenching, by whatever mechanism, would be complete when the molecule entered a membrane-enclosed volume.

(3) Intrinsic fluorescence of the probe would be independent of the composition of the external medium.

(4) The probe would be capable of rapid equilibration across membranes in response to pH gradients.

In the discussion to follow, we will compare atebrin and 9-aminoacridine in light of the above considerations.

Measurements of quenching and estimation of A_i/A_0

When atebrin and 9-aminoacridine were exposed to pigmented liposome membranes enclosing a phase at a pH lower than that of the external medium, the fluorescence of the amines was quenched. In the absence of pigment, atebrin fluorescence was enhanced. If these changes in fluorescence were related to pH gradients, it would be expected that discharging the gradient would reverse the change. Triton X-100 is a non-ionic detergent which releases ion gradients by causing membranes to become generally permeable to ions[16]. NH_4Cl is a monoamine which interacts with pH gradients according to Eqn 1 below. If present in sufficient concentration, it follows that NH_4Cl and other amines would equilibrate with a pH gradient and greatly reduce its magnitude[17]. Addition of either Triton or NH_4Cl reversed the fluorescence changes described above and we concluded that the changes were dependent on pH gradients across the liposome membranes.

The value A_i/A_0 was measured by assuming that fluorescence was completely quenched when atebrin or 9-aminoacridine entered a membrane-enclosed volume. For instance, fluorescence in the presence of liposomes might be 50 arbitrary units, and then increase to 100 units upon Triton addition. We would assume that half the amine had been in the liposomes, and that $A_i/A_0 = 1.0$. It was necessary to use the fluorescence level following Triton addition as a baseline, since some of the components of the system (bacteriochlorophyll, ascorbate, ferricyanide) could partially quench fluorescence (see Fig. 2).

Monoamines and diamines

9-Aminoacridine is a monoamine with pK = 10.0 (ref. 3). Ideally it should distribute across membranes[3] according to the relation

$$\frac{[A^T]_i}{[A^T]_o} = \frac{K_a + [H^+]_i}{K_a + [H^+]_o} \tag{1}$$

where $[A^T]$ is the total concentration of the amine, K_a is the dissociation constant of the amine and $[H^+]$ is the concentration of protons. When K_a is small relative to $[H^+]$ this relation may be expressed in logarithmic form as

$$\log \frac{A_i}{A_o} + \log \frac{V_o}{V_i} = -\Delta pH_{(i-o)} \tag{2}$$

where V_o/V_i is the volume ratio of the external volume to the internal membrane enclosed volume, and A_i and A_o are the total amine in the internal and external volumes, respectively. We could test this relation in the liposome system, even though values of V_o/V_i were unknown, by varying ΔpH for several lipid concentrations. The data could then be compared with theoretical lines assuming that one set of data points was correct. When this was carried out, we found that 9-aminoacridine behaved as an ideal monoamine when lipid concentration ranged from 0.06 to 0.24 mg/ml.

Atebrin is a diamine (pK_1 = 7.9, pK_2 = 10.5), which should ideally distribute across membranes according to the relation.

$$\frac{[A^T]_i}{[A^T]_o} = \frac{K_1K_2 + K_1[H^+]_i + [H^+]_i^2}{K_1K_2 + K_1[H^+]_o + [H^+]_o^2} \tag{3}$$

For atebrin at $[H^+]_i > 10^{-6}$, this simplifies to

$$\frac{[A^T]_i}{[A^T]_o} = \frac{[H^+]_i^2}{K_1[H^+]_o + [H^+]_o^2} \tag{4}$$

Atebrin was tested in the liposome system by graphing the logarithmic form of Eqn 4 and comparing the resulting line with actual data. Atebrin did not behave as an ideal diamine, and the experimental data deviated considerably from the above relation (Fig. 5). The reason for this deviation is unknown, but may be related to incomplete quenching, or the fluorescence enhancement we observed under certain conditions.

Fluorescence quenching and enhancement

The mechanism by which distribution of atebrin and 9-aminoacridine across membranes alters their fluorescence is unknown. There are several possible effects which may contribute to the observed quenching:

(1) *Screening.* If a fluorescent molecule disappears from an external volume and enters a volume which is shielded by a pigment layer which in turn absorbs in the excitation or emission wavelength, it may be imagined that some quenching of fluorescence may occur. Schuldiner and Avron[18] suggested that this may be the major effect when atebrin fluorescence is quenched by illuminated chloroplasts. However, it is not clear that the very thin layer of pigment which separates the external and inter-

nal volumes in the liposome system would provide sufficient absorption to account for all the observed quenching. Furthermore, this mechanism certainly cannot account for the 9-aminoacridine quenching which does not depend on the presence of pigment.

(2) *Effect of pH on intrinsic fluorescence.* Atebrin fluorescence decreases by half as it accepts a second proton at lower pH ranges (Fig. 1) and this may account for a certain amount of quenching, as will be discussed later. Quenching of 9-aminoacridine fluorescence cannot be accounted for in this way.

(3) *Self-quenching.* Energy transfer between molecules of the same species may cause a decreased quantum yield of fluorescence at a specified emission wavelength. The extent of the self-quenching is naturally concentration dependent, and it may be imagined that when a fluorescent amine is concentrated within liposomes self-quenching may become important. It should be noted that under conditions described here, quite high concentrations of the amine would build up within the membrane enclosed volume. For instance, in Fig. 4, the apparent values for V_0/V_i obtained from the intercepts of the three largest lipid concentrations are 200, 400 and 800. The amine concentrations inside for $A_i/A_0 = 1$ would therefore be 0.4, 0.8 and 1.6 mM, respectively.

(4) *Interaction with other molecules.* A fluorescent molecule may also lose its energy by interaction with other molecules. For instance, this is clearly evident in the quenching of 9-aminoacridine fluorescence by organic buffers (Fig. 1). A similar effect may contribute to quenching if the amine interacted with the components of liposomal or other membranes.

Comparison of atebrin and 9-aminoacridine

The fact that fluorescence is enhanced when atebrin is concentrated in liposomes further complicates the interpretation of results when it is used as a pH probe. Fig. 3 shows the enhancement effect over a range of pH. The enhancement, which was maximal at ΔpH of 1–2 pH units, decreased at larger pH values. This may be understood from the pH dependence of intrinsic atebrin fluorescence (Fig. 1). An atebrin molecule at pH 8 has twice the intrinsic fluorescence that it has at pH 5–6. Thus, atebrin leaving an external environment at pH 8 and entering an internal volume at pH 5 would lose half its fluorescence, and this would overcome the enhancement effect. The enhancement itself may possibly result from "excimer" formation as atebrin becomes more concentrated[19].

If bacteriochlorophyll or other pigments (chlorophyll *a*, *b* and carotene) were included in the liposome membranes, atebrin fluorescence was quenched. The quenching was maximal at 2–3 mole % bacteriochlorophyll, but it was not certain that the quenching was complete even with the pigments present. 9-Aminoacridine fluorescence, in contrast to atebrin, was quenched in liposomes lacking pigments. This quenching was still incomplete, since addition of pigment produced further quenching (Fig. 2). Quenching of 9-aminoacridine fluorescence may be best understood as a concentration-dependent self-quenching, with an additional contribution to quenching by any pigments present in the membranes. The close fit of experimental results to the theoretical lines in Fig. 4 suggests that quenching in the presence of pigment was nearly complete.

We conclude that the monoamine, 9-aminoacridine, is superior to atebrin as a fluorescent indicator for ΔpH = 2–4 units. However, its fluorescence is partially

quenched by organic buffers, and the method is only slightly responsive to $\Delta pH <$ 2 pH units at volume ratios commonly used in experimental systems. These defects are far outweighed by its behaviour as an ideal monoamine as indicated by its response in the liposome system, and 9-aminoacridine should be quite useful for estimating ΔpH in membranous systems which develop acid interiors. Atebrin, although more responsive in the 0–2 ΔpH range, does not behave as an ideal diamine and displays an anomalous fluorescence enhancement in the liposome system. In a naturally pigmented system such as chloroplasts or chromatophores with high pigment concentration, atebrin fluorescence quenching may very well be complete. In a non-pigmented system, such as microsomes or mitochondria, atebrin probably could not be used effectively as a quantitative pH probe. It is interesting to note in this regard that Azzi *et al.*[10] found that atebrin fluorescence was partially quenched by submitochondrial particles during electron transport. It was suggested that the quenching may be related to an exchange of cationic atebrin for protons across the membranes. However, in view of the previous investigations with chloroplasts and the present study with liposomes, it seems more probably that atebrin was simply moving inward, with a consequent partial fluorescence quenching in response to a pH gradient developing across the submitochondrial membranes.

Estimation of V_0/V_i

Calculations of ΔpH from a theoretical consideration of amine distribution depend on knowing the ratio V_0/V_i. We were not able to make a reliable estimate of this ratio in the present liposome system. The usual method of finding sucrose or inulin impermeable space was impractical, since the liposomes did not form a centrifugal pellet under the conditions required for establishing pH gradients.

However, exact values of V_0/V_i are unnecessary if the data shown in Fig. 4 are used as a calibration curve. For any given lipid concentration, one may assume that under similar ionic and osmotic conditions, the V_0/V_i ratio does not vary appreciably. We were therefore able to estimate ΔpH in the redox couple and nigericin systems described below, since the lipid concentrations were within the range shown in Fig. 4.

Development of pH gradients across liposomal membranes

Redox couples and carrier molecules

The presumed reaction sequence for the redox reaction established across liposomal membranes is shown in Fig. 8.

The midpoint potential (E_0^1(pH 7)) for the ascorbate couple is + 60 mV, and for ferro/ferricyanide is + 430 mV. In the liposome system described here containing 1 mM ascorbate and 0.03 mM ferricyanide (total concentration), the ferricyanide would essentially be completely reduced if the reaction occurred in free solution.

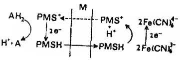


Fig. 8. Proposed reaction sequence for development of pH gradients by coupled redox reactions across liposome membranes. See text for details.

However, since the couples were separated by proton-impermeable membranes, a pH gradient would build up which would ultimately come to equilibrium with the driving force of the downhill electron transport reaction. It is difficult to calculate the relative concentrations at which this might occur. In one experiment the amount of ferricyanide reacting under the usual conditions was measured in a dual wavelength spectrophotometer, and it was found that approximately half the ferricyanide present was quickly reduced. Since the original concentration of ferricyanide in the liposomes was 0.1 M, this would suggest that about 0.05 M protons might be produced within the liposomes, equivalent to pH 1.3. At pH 7 outside, a ΔpH of 5 pH units or more would result. The 9-aminoacridine method could not accurately measure a pH gradient of this magnitude, but certainly the quenching of 9-aminoacridine fluorescence would indicate that a gradient of at least 4 pH units developed under the above conditions.

If ferrocyanide was used as a reductant, rather than ascorbate, the final equilibrium depended only on the relative concentrations of ferro- and ferricyanide in the system. Since the redox potential difference at the concentrations used was small relative to the ascorbate–ferricyanide system, one would expect a much smaller pH gradient to develop, and in fact the data for 9-aminoacridine quenching indicate a pH gradient of only 1.6 units.

The liposome system offers a potential model system for studying the mechanism of electron transport across membranes. For instance, the results with PMS (Fig. 6C) suggest that there are two steps in which PMS crosses the membranes. The first of these is the inward transport of electrons by PMS in its reduced, uncharged form, and is quite rapid. The second is the much slower, outward diffusion of PMS^+ across the membranes Thus, we only see a rapid development of a pH gradient if PMS is present in concentrations approaching the total amount of ferricyanide in the system (Fig. 6A). If smaller amounts are used, the second step becomes rate limiting (Fig. 6C). In preliminary experiments (R.C. Prince, unpublished) other carriers such as diaminodurol and dichlorophenolindophenol act at catalytic concentrations suggesting that both oxidized and reduced forms of these dyes readily cross the membrane.

Proton–K^+ exchange mediated by nigericin

The pH gradient developed when K^+ exchanges for protons in the liposome systems depends simply on the relative concentrations of K^+ across the membranes, as shown in Fig. 7. Under these controlled conditions, a ΔpH of 2.2 units as estimated from the calibration curve in Fig. 4 may be established when the expected $K_i^+/K_o^+ = 200$. The fact that 9-aminoacridine behaves ideally in the nigericin system is additional support for the conclusion that it may be used to quantitate pH gradients with considerable accuracy.

ACKNOWLEDEGEMENTS

The authors wish to thank A. D. Bangham, N. Good, J. D. McGivan, B. A. Melandri and R. Cogdell for helpful discussion during the course of this study, and gratefully acknowledge financial assistance from the Science Research Council for equipment and support (R. C. P.). One of us (D. W. D.) was the recipient of a Wellcome Foundation Travel Grant.

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EXHIBIT C

Vol. 75, No. 2, 1977 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

NMR STUDIES OF pH-INDUCED TRANSPORT OF CARBOXYLIC ACIDS ACROSS PHOSPHOLIPID VESICLE MEMBRANES[1]

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Received January 31,1977

SUMMARY

Proton NMR spectroscopy was used to demonstrate that transmembrane pH gradients across single-bilayer vesicle membranes effect the transport and concentration of carboxylic acids. The results obtained indicate that this transport occurs <u>via</u> selective permeation of the membrane by the protonated (uncharged) form of the acid.

INTRODUCTION

The active transport across biological membranes of small hydrophilic molecules is important to the metabolic and regulatory functions of the cell. The possible mechanisms for accomplishing this transport are quite varied. One of the simplest, however, utilizes a pH gradient to directly raise the chemical potential of a permeable form of a titratable solute on one side of a membrane, resulting in the passive diffusion of the solute. It is widely accepted that the neutral form of a molecule, such as a carboxylic acid, is more lipid soluble and hence more permeable than its ionic counterparts. Thus, lowering the pH of a carboxylic acid solution, which is external to a closed vesicular membrane, should concentrate the acid in the interior volume of the vesicle. An analogous mechanism has been proposed for the concentra-

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[1]Preliminary results were reported at the 172nd American Chemical Society meeting, San Francisco, California, August 29-September 3, 1976.

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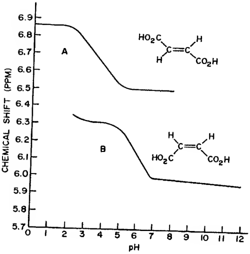

Figure 1. Chemical shift titration curve for the vinyl protons of fumaric (A) and maleic (B) acids. Curve A is taken from work reported by L. Pratt and B. B. Smith (3) and is for a 0.04 M aqueous solution containing t-butanol as an internal standard (δ = 1.25). The shifts for curve B are referenced to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (δ = 0.0) and are for an 8.6 mM D_2O solution containing phosphatidylcholine-cholesterol vesicles.

tion of catecholamines within chromaffin vesicles (1) and has led to recent studies of the correlation of pH gradients with transport phenomena (2).

It is often not a simple task to quickly and accurately obtain pH and concentration information for solutions external and internal to vesicular structures less than 1000 Å in diameter. High field proton NMR offers a means of accomplishing this for many systems. We report here an illustration of this fact, using the pH-induced transport across phospholipid vesicles of fumaric and maleic acids. These acids exhibit vinyl proton resonances which are resolved from vesicle absorptions and which exhibit a strong chemical shift dependence on pH as shown in Figure 1.

MATERIALS AND METHODS

Single-bilayer 320 Å diameter vesicles containing maleate dianion were prepared by sonicating a 10% (w/v) egg yolk phosphatidylcholine-cholesterol (2:1 mole ratio) suspension in a 0.19 M maleic acid solution in D_2O at pH 7. The exterior maleate solution was replaced by a pH 7 equiosmolar D_2O solution

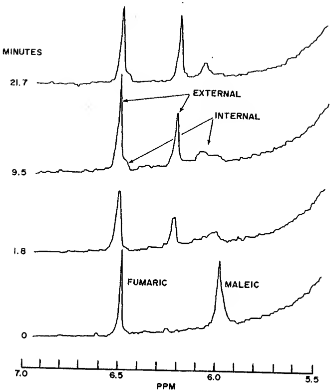

Figure 2. Vinyl proton NMR spectra recorded at various times after a transmembrane pH gradient was established by lowering the extravesicular pH to 5.5.

containing fumarate dianion (9.3 mM), NaCl (0.26 M), and sodium 2,2-dimethyl-2-silapentane-5-sulfonate (4.6 mM) which served as an internal NMR reference. This solution replacement was accomplished by passing the vesicle preparation through a Sephadex G-50 column, which had previously been equilibrated with the above fumarate solution.

A pH gradient was established across the vesicle membrane by lowering the exterior pH through the addition of 0.4 N DCl. Sequential FT NMR measurements were then made with a Bruker HX270 spectrometer to monitor the pH and carboxylic acid concentrations for both the exterior and interior regions of the vesicle preparation.

RESULTS

Figure 2 presents a series of spectra observed when the external pH was lowered to 5.5. Lowering the outside pH serves to drive the transport of external fumaric acid into the interior of the vesicles. This is demonstrated by the growth of an inside fumaric acid peak, which appears as a high field shoulder on the external acid resonance at 6.50 ppm. The gradual downfield

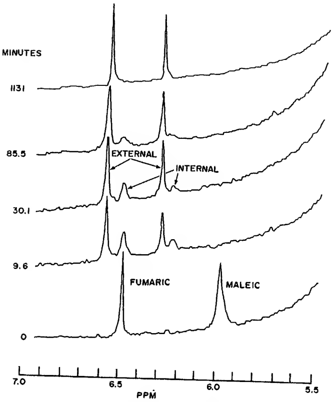

Figure 3. Vinyl proton NMR spectra recorded at various times after a transmembrane pH gradient was established by lowering the extravesicular pH to 4.7.

shift of the internal maleic acid peak from 6.0 to 6.1 ppm, in concert with the growth of the inside fumaric acid signal, indicates that fumaric acid is being transported with protons, resulting in the titration of maleate dianion. The broad appearance of the inside maleic acid signal at 1.8 and 9.5 minutes indicates that a range of internal pH values exists, which probably reflects the inhomogeneity of the vesicle preparation with respect to permeability and size.

Figures 3 and 4 reveal that adjusting the outside pH to a lower value (4.7 compared to 5.5) results in a rapid and greater accumulation of internal fumaric acid, followed by a slow simultaneous leakage of both acids. Note, in contrast to the case just considered, the inside fumaric acid peak in

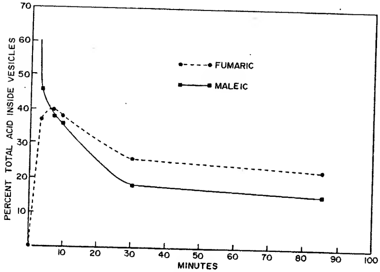

Figure 4. Time variation of internal acid concentrations for data presented in Figure 3.

Figure 3 is clearly resolved from the outside signal in accord with the increased chemical shift dependence on pH at pH values less than 5, as shown in Figure 1. Figure 4 demonstrates that the pH gradient in this case drives the transport of 40% of the total fumaric acid. This corresponds to internal and external concentrations of 74 mM and 5.9 mM, respectively, if one assumes the internal volume of the vesicle preparation to be 5% of the total volume. Thus, in this instance the pH gradient serves to concentrate fumaric acid by a factor of 13.

DISCUSSION

Fumaric and maleic acids exist as neutral molecules (H_2A), monoanions (HA^-), or dianions ($A^=$). On the basis of the passive diffusion model presented earlier, these acids would be predicted to undergo preferential transport in their more lipid-soluble fully protonated forms (H_2A). This would result in a tight coupling of proton and carboxylate transport. On this basis we predict that, following a pH perturbation, an equilibrium con-

dition, corresponding to zero net transport, should be reached where the internal and external H_2A activities are equal.

Since activities in these systems are difficult to predict, we will deal here only with concentrations. The H_2A concentration for dicarboxylic acids may be expressed as a function of total acid concentration (C_T), pH, and ionization constants (K_1 and K_2) as shown in Equation 1.

$$[H_2A] = \frac{C_T \times 10^{-2pH}}{10^{-2pH} + K_1 \times 10^{-pH} + K_1K_2} \quad (1)$$

Calculation of H_2A concentration for fumaric acid using the observed pH values and concentrations, corresponding to maximal internal accumulation in Figure 4, yields 5×10^{-5} M and 4×10^{-5} M for inside and outside acid, respectively. These values are within experimental error of being equivalent and therefore offer direct evidence for the coupling of proton and carboxylate transport via the selective transport of the fully protonated acid form.

On the basis of this coupled transport scheme, the initial rate of fumaric acid accumulation, in the experiments we have described, should be a linear function of the initial concentration of the H_2A species. Since the concentration of H_2A is a strong function of pH, we would expect more rapid initial transport in the experiment described by Figures 3 and 4, where the outside pH was lowered to 4.7, compared to the case in Figure 2 where the pH was only lowered to 5.5. On the basis of Equation 1 the concentration of H_2A is 16 times greater at pH 4.7 than at pH 5.5. Initial transport rates observed in these experiments, although based on a small number of data points, are consistent with this factor and thus provide further support for the transport mechanism discussed above.

The nonselective leakage of both fumaric and maleic acids depicted in Figure 4 at longer times is probably the result of vesicle rupture in response to the osmotic stress provided by the early selective transport of protonated

fumaric acid. This contention is supported by our observation of enhanced leakage of normally impermeant $N(CH_3)_4^+$, when $N(CH_3)_4Cl$ is included in the vesicle preparation.

The NMR spectra in this study were acquired in less than 70 seconds in FT mode. The spectrometer used has the capability of accumulating sequential spectra in a computer automated manner, thereby greatly facilitating the study of rapid transport phenomena. The use of other NMR kinetic procedures and the use of vesicles of differing size can further extend the transport time scales which are amenable to study. The variety of solutes yielding resolvable and pH sensitive resonances is large and promises a variety of applications in the future.

ACKNOWLEDGMENT

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EXHIBIT D

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/759,222	01/20/2004	Rolf Joachim Mehlhorn	028723-389	6448

7590 09/05/2008

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ART UNIT	PAPER NUMBER
1614	

MAIL DATE	DELIVERY MODE
09/05/2008	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	10/759,222	MEHLHORN, ROLF JOACHIM	
	Examiner	Art Unit	
	Kevin E. Weddington	1614	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *29 May 2008*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-26* is/are pending in the application.

4a) Of the above claim(s) *13-15 and 24-26* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-12 and 16-23* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

Claims 1-26 are presented for examination.

Applicants' amendment and response filed May 29, 2008 have been received and entered.

Accordingly, the rejection made under 35 USC 112, second paragraph as set forth in the previous Office action dated November 29, 2007 at page 5 is hereby withdrawn because the applicants rewrote the claims for clarity.

Accordingly, the rejections made under 35 USC 102(b) as being anticipated by Nichols et al., Deamer et al., and Cramer et al. as set forth in the previous Office action dated November 29, 2007 at pages 5-6 are hereby withdrawn because of applicants' remarks.

Claims 13-15 and 24-26 are withdrawn form consideration as being drawn to non-elected invention (37 CFR1.142(b)).

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 16-23 are again rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-7 of U.S. Patent No. 5,762,957. Although the conflicting claims are not identical, they are not patentably distinct from each other because of record, for reason of record as set forth in the previous Office action dated November 29, 2007 at pages 2-3 as applied to claims 16-23 is **MAINTAINED**.

Applicants' remarks regarding the patented application does not teach the impermeability of its liposome membrane to the buffer or to the selection of a first solution pH so as to the result in stable vesicles and buffers for at least one week; or specify any particular pH are not persuasive since the present application and the patented application are claiming the same components in both kits. Thus the two applications' components in their respective kits would produce the same results. Note in column 3, lines 13-15 of the patented application discloses the first solution has a selected pH such that the stability of the vesicle and its buffer will be maintained for a period of at least one week.

The rejection made under obviousness-type double patenting is adhered to.

Claims 16-23 are not allowed.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims

are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 1-12 are again rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-9 of U.S. Patent No. 5,827,532. Although the conflicting claims are not identical, they are not patentably distinct from each other because of record, for reason of record as set forth in the previous Office action dated November 29, 2007 at pages 3-4 as applied to claims 1-12 is **MAINTAINED**.

Applicants stated on page 14 of the response that a terminal disclaimer was submitted herewith; however, no such terminal disclaimer was submitted.

The rejection made under obviousness-type double patenting is adhered to.

Claims 1-12 are not allowed.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 1-8, 10-12 and 16-23 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

This is a written description rejection.

A lack of adequate written description issue arises if the knowledge and level of skill in the art would not permit one skilled in the art to immediately envisage the product claimed from the disclosed process. See, e.g., Fujikawa v. Wattanasin, 93 F.3d 1559, 1571, 39 USPQ2d 1895, 1905 (Fed. Cir. 1996) (a "laundry list" disclosure of every possible moiety does not constitute a written description of every species in a genus because it would not "reasonably lead" those skilled in the art to any particular species); In re Ruschig, 379 F.2d 990, 995, 154 USPQ 118, 123 (CCPA 1967).

An applicant may also show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics which provide evidence that applicant was in possession of the claimed invention, i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.

In particular, the specification as original filed fails to provide sufficient written bases of any of the agents demonstrating wherein possession of use of the broad terms: **a chemical species, an amino group, an amine, a drug, a first substance, and a**

second substance. The mere fact that Applicant may have discovered one type of chemical species may be loaded into the lipid-like vesicles and achieve the desire stability is not sufficient to claim the entire genus.

The written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice, reduction to drawings, or by disclosure of relevant, identifying characteristics, i.e., structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus. See Eli Lilly, 119 F.3d at 1568, 43 USPQ2d at 1406.

A "representative number of species" means that the species which are adequately described are representative of the entire genus. Thus, when there is substantial variation within the genus, one must describe a sufficient variety of species to reflect the variation within the genus. The disclosure of only one species encompassed within a genus adequately describes a claim directed to that genus only if the disclosure "indicates that the patentee has invented species sufficient to constitute the gen[us]."

Claims 1-8, 10-12 and 16-23 are not allowed.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the

invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

The factual inquiries set forth in *Graham* **v.** *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Claims 1-12 are again rejected under 35 U.S.C. 103(a) as being unpatentable over Nichols et al. or Deamer et al. or Cramer et al.

Applicants' remarks regarding the prior art, each individual reference, do not teach one skilled in the chemistry art to establish a pH gradient when an internal medium is basic one should add an acid to the external medium are not persuasive since the prior art, Nichols et al., Deamer et al. and Cramer et al., does teach the concept of loading a chemical species into the liposomes using a pH gradient. Again, it

would have been obvious to one of ordinary skill in the art to load any drug with the expectation of similar loading since applicants have not demonstrated side-by-side comparison of the prior art's liposome loading versus the present application's liposome loading.

The rejection made under 35 USC 103(a) is adhered to.

Claims 1-12 are not allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Kevin E. Weddington whose telephone number is (571)272-0587. The examiner can normally be reached on 12:30 pm-9:00 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Ardin Marschel can be reached on (571)272-0718. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

Kevin E. Weddington

Primary Examiner
Art Unit 1614

/Kevin E. Weddington/
Primary Examiner, Art Unit 1614

EXHIBIT E

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P O Box 1450
Alexandria, Virginia 22313-1450
www uspto gov

APPLICATION NO	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO	CONFIRMATION NO.
10/759,222	01/20/2004	Rolf Joachim Mehlhorn	028723-389	6448

7590 03/31/2009

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EXAMINER	
WEDDINGTON, KEVIN E	

ART UNIT	PAPER NUMBER
1614	

MAIL DATE	DELIVERY MODE
03/31/2009	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	10/759,222	MEHLHORN, ROLF JOACHIM	
	Examiner	Art Unit	
	Kevin E. Weddington	1614	

-- *The MAILING DATE of this communication appears on the cover sheet with the correspondence address* --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *05 January 2009*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-26* is/are pending in the application.

4a) Of the above claim(s) *13-15 and 24-26* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-12 and 16-23* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)

3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.

4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

5) ☐ Notice of Informal Patent Application

6) ☐ Other: _____.

Claims 1-26 are presented for examination.

Applicants' request for reconsideration and terminal disclaimer filed January 5, 2009 have been received and entered.

Accordingly, the rejection made under obviousness-type double patenting over claims 1-9 of U. S. Patent No. 5,827,532 as set forth in the previous Office action dated September 5, 2008 at pages 3-4 is hereby withdrawn because the applicants filed a terminal disclaimer.

Claims 13-15 and 24-26 are withdrawn from consideration as being drawn to the non-elected invention (37 CFR 1.142(b)).

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 16-23 are again rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-7 of U.S. Patent No. 5,762,957. Although the conflicting claims are not identical, they are not patentably distinct from each other because of record, for reason of record as set forth in the previous Office action dated September 5, 2008 at pages 2-3 as applied to claims 16-23 is hereby **MAINTAINED**.

Applicants' remarks regarding the kit of the present application is different from the kit of the patent application are not persuasive since in the patented application in column 3, lines 13-15 teaches the first solution has a selected pH such that the stability of the vesicle and its buffer will be maintained for a period of at least one week. The patented application mentions that the mechanism of the present application's kit; therefore, this mechanism is suggested and covered by the patented application.

The rejection made under obviousness-type double patent is adhered to.

Claims 16-23 are not allowed.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 1-8, 10-12 and 16-23 are again rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to

reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

This is a written description rejection.

A lack of adequate written description issue arises if the knowledge and level of skill in the art would not permit one skilled in the art to immediately envisage the product claimed from the disclosed process. See, e.g., Fujikawa v. Wattanasin, 93 F.3d 1559, 1571, 39 USPQ2d 1895, 1905 (Fed. Cir. 1996) (a "laundry list" disclosure of every possible moiety does not constitute a written description of every species in a genus because it would not "reasonably lead" those skilled in the art to any particular species); In re Ruschig, 379 F.2d 990, 995, 154 USPQ 118, 123 (CCPA 1967).

An applicant may also show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics which provide evidence that applicant was in possession of the claimed invention, i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.

In particular, the specification as original filed fails to provide sufficient written bases of any of the agents demonstrating wherein possession of use of the broad terms: **a chemical species, an amino group, an amine, a drug, a first substance, and a second substance**. The mere fact that Applicant may have discovered one type of chemical species may be loaded into the lipid-like vesicles and achieve the desired stability is not sufficient to claim the entire genus.

The written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice, reduction to drawings, or by disclosure of relevant, identifying characteristics, i.e., structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus. See Eli Lilly, 119 F.3d at 1568, 43 USPQ2d at 1406.

A "representative number of species" means that the species which are adequately described are representative of the entire genus. Thus, when there is substantial variation within the genus, one must describe a sufficient variety of species to reflect the variation within the genus. The disclosure of only one species encompassed within a genus adequately describes a claim directed to that genus only if the disclosure "indicates that the patentee has invented species sufficient to constitute the gen[us]."

Applicants' remarks regarding the above broad terms are supported by the instant specification and the applicants are not laying claim to certain chemical species, amines, drugs and the like are not persuasive because how does the instant invention works if no specific chemical species, amine, drug or the like is used to produce the finished product. So any chemical species can produce the instant invention.

Claims 1-8, 10-12 and 16-23 are not allowed.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

The factual inquiries set forth in *Graham* v. *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Claims 1-12 are again rejected under 35 U.S.C. 103(a) as being unpatentable over Nichols et al., (C46 of PTO-1449), Deamer et al. (C15 of PTO-1449), or Cramer et al. (C7 of PTO-1449), all of record, for reasons of record as set forth in the previous Office action dated September 5, 2008 at pages 6-8 as applied to claims 1-12.

Again, applicants' remarks regarding the prior art, each individual reference, does not teach one skilled in the chemistry art to establish a pH gradient when an internal medium is basic, one should add an acid to the external medium are not persuasive since the prior art, Nichols et al., Deamer et al, and Cramer et al., does teach the concept of loading a chemical species into the liposomes using a pH gradient. Again, it would have been obvious to one of ordinary skill in the art to load any drug the expectation of similar loading since applicants have not demonstrated on the record any side-by-side comparison of the prior art's liposome loading versus the present application's liposome loading.

The rejection made under 3 5USC 103(a) is adhered to.

Claims 1-12 are not allowed.

Conclusion

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Kevin E. Weddington whose telephone number is (571)272-0587. The examiner can normally be reached on 12:30 pm-9:00 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Ardin Marschel can be reached on (571)272-0718. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

Kevin E. Weddington
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EXHIBIT F

US 20040208922A1

(19) United States
(12) Patent Application Publication (10) Pub. No.: US 2004/0208922 A1
Mehlhorn (43) Pub. Date: Oct. 21, 2004

(54) METHOD FOR LOADING LIPID LIKE VESICLES WITH DRUGS OR OTHER CHEMICALS

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(73) Assignee: The Regents of the University of California, Oakland, CA (US)

(21) Appl. No.: 10/759,222

(22) Filed: Jan. 20, 2004

Related U.S. Application Data

(63) Continuation of application No. 08/472,843, filed on Jun. 7, 1995, now abandoned.

Publication Classification

(51) Int. Cl.^{7} A61K 9/127

(52) U.S. Cl. 424/450

(57) ABSTRACT

A method for accumulating drugs or other chemicals within synthetic, lipid-like vesicles by means of a pH gradient imposed on the vesicles just prior to use is described. The method is suited for accumulating molecules with basic or acid moieties which are permeable to the vessels membranes in their uncharged form and for molecules that contain charge moieties that are hydrophobic ions and can therefore cross the vesicle membranes in their charged form.

The method is advantageous over prior art methods for encapsulating biologically active materials within vesicles in that it achieves very high degrees of loading with simple procedures that are economical and require little technical expertise, furthermore kits which can be stored for prolonged periods prior to use without impairment of the capacity to achieve drug accumulation are described.

A related application of the method consists of using this technology to detoxify animals that have been exposed to poisons with basic, weak acid or hydrophobic charge groups within their molecular structures.

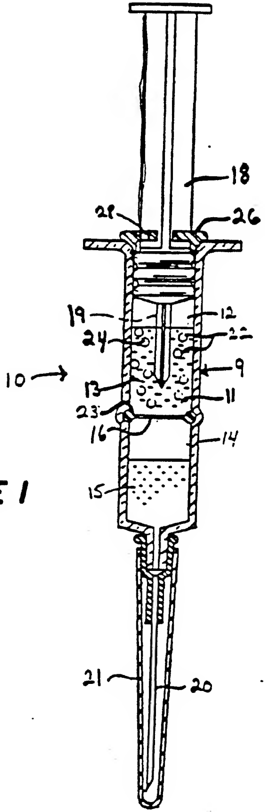

FIGURE 1

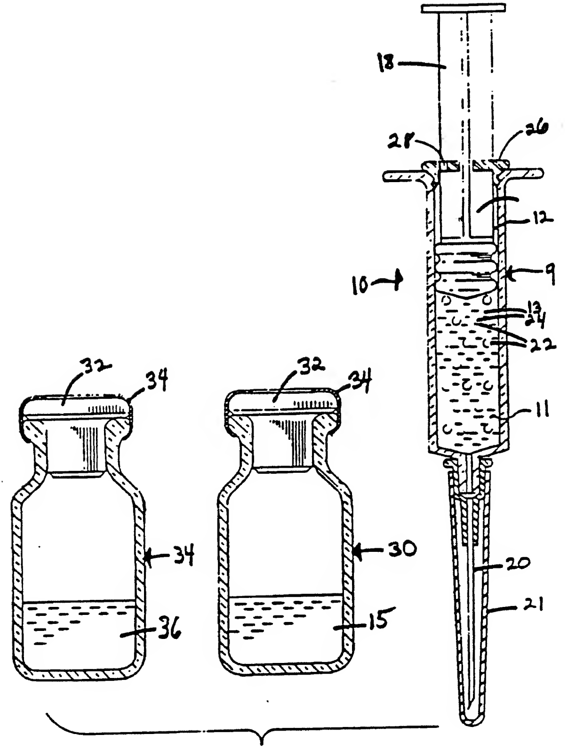

FIGURE 2

METHOD FOR LOADING LIPID LIKE VESICLES WITH DRUGS OR OTHER CHEMICALS

ACKNOWLEDGEMENT OF GOVERNMENT SUPPORT

[0001] This invention was made with Government support under Grant No: DE-AC03-76SF00093 with the Department of Energy and the University of California. The Government bas the rigbts in this invention.

TECHNICAL FIELD

[0002] The invention relates to a method for loading lipid-like vesicles with a drug or other chemical species by establishing a preimposed pH gradient.

BACKGROUND ART

[0003] The use of membranous vesicles such as liposomes and the like as adjuvants and carriers for drugs, other chemicals and biologically active compounds such as antigens and antibodies is well known in the field (U.S. Pat. Nos. 4,053,585; 4,397,846; 4,411,894; 4,427,649).

[0004] Also, many methods exist to encapsulate the various drugs or other chemicals within the vesicles. U.S. Pat. No. 4,241,046, discloses a method for encapsulating biologically active materials within liposomes by providing a combination of lipids in an organic solvent and an aqueous mixture of the material for encapsulation, emulsifying the provided mixture, removing the organic solvent, and suspending the resulting gel in water. The biologically active material is encapsulated by being processed with the liposome during preparation of the liposome.

[0005] U.S. Pat. No. 3,804,776 discloses a method for producing oil and fat encapsulated amino acids or polypeptides by dispersion. Powders of the material desired to be encapsulated are combined in a molten mixture of the lipid material. Thereafter tbe molten mixture is poured into water. This method of encapsulation, however, only allows for oral administration of the encapsulated material, since the droplets of lipids enclosing the encapsulated material are too large to be delivered parenterally.

[0006] Most of the other known methods also involve encapsulating the desired drug or other chemical during the synthesis of the liposomes (Papahadjopoulos, et al., *Biochim., Biophys. Acta,* 135:639 (1967); Bangham et al., *J. Mol. Biol.,* 12:238-2S2 (1965); and Bapzri and Korn, *Biochim., Biophys. Acta,* 298:1015 (1973)). All of the methods described, either employ laborious procedures requiring skill and training or the use of sophisticated and expensive equipment, have a low efficiency of encapsulation or low encapsulation rate or involve encapsulating the drug simultaneously with tbe preparation of the vesicle, thereby invoking possible leakage of tbc encapsulated chemical. Also these methods leave a substantial portion of the substance sequestered outside of the vesicle since at best only 50% enclosed volumes of tbe encapsulated material relative to total volumes of the vesicles have been reported. Tbese methods therefore require that expensive drugs used for encapsulation be recovered from the drug solution in which the vesicles were prepared. The prior art field of encapsulation methods thus has a number of very serious problems

DISCLOSURE OF INVENTION

[0007] The present invention is directed to overcoming one or more of the problems as set forth above.

[0008] In accordance with an embodiment of the present invention, a method is set out for loading lipid-like vesicles having a membrane permeable to a cbemical species to be loaded from a loading solution wherein tbe concentration of the loaded chemical species within the vesicle is greater than the concentration of the chemical species in the loading solution and the loaded chemical specimens can be substantially maintained within the vesicle for at least one-quarter hour following loading. The method comprises inducing a pH gradient across the vesicle membrane while the vesicle is in the loading solution containing the chemical species with the pH gradient having been selected to drive the chemical species into the vesicles.

[0009] In accordance with a second aspect of the present invention, a method is set out for loading lipid-like vesicles having a membrane permeable to a chemical species to be loaded and having the capability to maintain the loaded chemical species within the vesicle for at least one-quarter hour following loading by inducing a pH gradient across the membrane. The method comprises incorporating within the vesicle a buffer solution buffered to a selected acid or alkaline pH and having a selected molarity and at least one selected pKa approximately equal to the selected buffer pH. The membrane is substantially impermeable to the buffer for at least one-quarter hour following loading of the chemical species and the vesicles are positioned in a bulk solution having a selected pH. The term "solution" is sometimes used loosely in the application to indicate a suspension in instances where lipid-like vesicles are present (i.e. suspended) in a solution.

[0010] The bulk solution is provided with a chemical species which has one or more selected acid pH responsive groups (i.e. groups which titrate as a function of pH by losing a negative charge upon being protonated) if the buffer is alkaline or one or more basic pH responsive groups (i.e. groups which titrate as a function of pH by becoming positively charged upon being protonated) if the buffer is acidic. The pH of the bulk solution is respectively at least 0.5, 0.3 or 0.2 of a pH unit bigher tban tbe pH of tbe buffer if the buffer is acidic and the chemical species has respectively one, two, or three or more basic pH responsive groups. The pH of the bulk solution is at least respectively 0.5, 0.3 or 0.2 f a pH unit lower than th pH of the buffer if th buffer is basic and th chemical species has respectively one, two, or three or more acid pH responsive groups

[0011] The pH responsive groups of the chemical species having one or more acid pH responsive groups have a pKa that is generally lower than or equal to the pH of the bulk solution and generally higher than or equal to 3.5 and the pH responsive groups of the chemical species having one or more basic pH responsive groups bave a pH tbat is generally higher than or equal to the pH of the bulk solution and generally lower than or equal to 11.

[0012] In accordance with a third aspect of the present invention, a pharmaceutical preparation for administration in vivo to an animal is provided by the method of encapsulation described above. The chemical species in this instance is a drug. The osmolarity of the buffer within tbe

vesicles is within the physiological range of the animal, the vesicles are suspended for administration in the bulk solution, and the pH of the bulk solution is physiologically benign.

[0013] In accordance with another aspect of the present invention, a kit is provided for loading lipid-like vesicles that have a membrane permeable to the chemical species to be loaded. The kit comprises a first compartment that has a first solution. The first solution has lipid-like vesicles incorporating a buffer buffered to a selected acid or basic pH. The buffer has at least one selected pKa approximately equal to the selected buffer pH and a selected molarity and cannot substantially permeate the vesicle for at least one-quarter hour following loading of the chemical species. The first solution also has a selected pH such that the stability of the vesicle and its buffer will be sustained for a period of at least one week at 4° C. The kit also comprises a second compartment, separate from the first compartment. The second compartment has a second solution that has a selected pH. Also included in the kit is a chemical species permeable to the vesicle. The chemical species has one or more selected acid pH responsive groups having selected pKas if the buffer is basic or one or more basic pH responsive groups having selected pKas if the buffer is acidic. The pH of the second solution is such that a mixture of the first and second solution will have a pH at least respectively 0.5, 0.3 or 0.2 of a pH unit higher than the pH of the buffer if the buffer is acidic and the chemical species has respectively one, two or three or more basic pH responsive groups or a pH at least respectively 0.5, 0.3 or 0.2 of a pH unit lower than the pH of the buffer if the buffer is basic and the chemical species has respectively one, two or three or more acid pH responsive groups. The pH responsive groups of the chemical species having one or more acid pH responsive groups have a pKa that is generally lower than or equal to the pH of a mixture of the first and second solutions and generally higher than or equal to 3.5. The pH responsive groups of the chemical species having one or more basic pH responsive groups have a pKa that is generally higher than or equal to the pH of a mixture of the first and second solutions and generally lower than or equal to 11. The chemical species is initially present in either one or the other of the two solutions.

[0014] In accordance with still another aspect of the present invention, another kit is provided for loading lipid-like vesicles having a membrane permeable to a chemical species to be loaded, the chemical species having acid or basic pH responsive groups. The kit comprises a first compartment that has a first solution having membranous lipid vesicles incorporating a buffer buffered to a selected alkaline pH if the chemical species to be loaded has acid pH responsive group or a selected acid pH if the chemical species has basic pH responsive groups. The buffer has at least one selected pKa approximately equal to the selected buffer pH, a selected molarity and cannot substantially permeate the vesicle membrane for at least one-quarter hour following loading of the chemical species. The first solution has a selected pH such that the stability of the vesicle and its buffer will be maintained for a period of at least one week at 4° C.

[0015] The kit further comprises a second separate compartment having a first substance (a compound or a second solution etc.) which when combined with the first solution will adjust the pH of the first solution so as to provide a predetermined pH gradient between the buffer within the vesicle and the pH adjusted first solution that will drive the chemical species into the vesicles. The kit also includes a third separate compartment having a second substance (yet another compound or a third solution etc.) which when combined with the pH adjusted first solution will further change the pH of said pH adjusted first solution to a value physiologically benign with regard to the blood of a mammal.

[0016] In accordance with still another aspect of the present invention, a method is provided for detoxifying an animal suffering from an overdose of a chemical species permeable to liposomes, the chemical species having basic or acid pH responsive groups (functions). The method comprises injecting the animal with a substantially physiologically benign solution having large volumes of liposomes having a buffer solution buffered to a pH generally lower than or equal to 5.4 if the chemical species' functions are basic pH responsive groups (amine, etc.) and a pH generally higher than or equal to 9.4 if the functions of the chemical species are acid pH responsive groups (carboxyl, etc.). The buffer also has a selected molarity within the physiological range of the animal and a selected pKa. The buffer also should not substantially permeate the vesicles for at least one hour after injection.

[0017] In accordance with a still further aspect of the present invention, a method is set forth for loading lipid-like vesicles having a membrane permeable to a chemical species to be loaded and for substantially maintaining the loaded chemical species within the vesicle for at least one-quarter hour following loading by inducing a pH gradient across the membrane. The method comprises incorporating within the vesicle a buffer solution buffered to a selected acid or alkaline pH and having a selected molarity at least one selected pKa approximately equal to the selected pH. The membrane is substantially impermeable to the buffer for at least one-quarter hour following loading. The vesicles are positioned in a bulk solution having a selected pH of either 0.5 to 3 pH units lower or higher than the pH of the buffer thereby establishing a transmembrane electrical potential. The inside of the vesicle will be positively charged if the pH outside the vesicle is more acid than inside or the inside of the vesicle will be negatively charged if the pH outside the vesicle is more basic than inside. The bulk solution is provided with a chemical species having membrane-permeable negatively charged ions if the membrane charge within the vesicle is positive or membrane-permeable positively charged ions if the membrane charge within the vesicle is negative.

[0018] When operating in accordance with the various embodiments of the present invention, vesicles such as liposomes can be loaded with drugs or other chemicals by an untrained person who simply reads some accompanying instructions. Not only will the chemicals be encapsulated with a high degree of loading (since the concentration of the chemical in the vesicle is practically independent of the concentration of the chemical in the solution used to prepare the vesicles or the concentration of the chemical in the solution containing the vesicles), thereby allowing for maximum concentration of scarce and expensive chemicals, but the encapsulation can be done quickly and easily. Also, fear of degradation of the vesicles and leakage of the chemicals

prior to administration need not be a concern, since the chemicals are easily encapsulated in the vesicles usually just before use, and the vesicles containing the chemical can be immediately delivered without further purification or other treatment provided the solution containing the loaded vesicles is physiologically benign. Drugs that have deloterious general effects such as chemotherapeutic or immunosuppressant drugs may be encapsulated in this manner and used to treat specific tissues or calls. Because of the high rate of encapsulation and the efficiency of encapsulation, concern over the expense and scarcity of the chemotherapeutic drugs no longer need be as great since only insignificant quantities of the drug will rein in the loading solution following vesicle preparation. Drugs encapsulated in this manner are sequestered within the vesicles (e.g., liposomes) until they reach the desired target tissue and are released when the membrane starts to break down and the drug begins to leak at the site of the desired tissue. (A process usually caused by lysosomal activity.)

BRIEF DESCRIPTION OF DRAWINGS

[0019] **FIG. 1** illustrates an elevational view of an embodiment of the invention as contemplated herein.

[0020] **FIG. 2** illustrates an elevational view of another embodiment of the invention.

DETAILED DESCRIPTION OF THE INVENTION

[0021] In accordance with aspects of the present invention, a method and kits are provided for quickly and efficiently loading vesicles have a membrane permeable to a chemical species having one or more selected acid pH responsive groups or basic pH responsive groups by inducing a pH gradient across the membrane of the vesicle. The vesicles contain a buffer solution buffered to a selected acid pH if the pH responsive groups of the chemical species are basic or an basic pH if the pH responsive groups of the chemical species or drug are acid.

[0022] The movement of many molecules across a vesicle membrane involves proton gradients (pH gradients) as the driving force (Rottenberg, H., "The Measurement of Membran Potential and A pH in Cells Organelles, and Vesicles", *Meth. Enzymol,* 55:547-569 (1979), Reinhold, L. and A. Kaplan, "Membrane Transport f Sugars and Amino Acids", *Ann. Rev. Plant Physiol.,* 35:45-83 (1984)). Electron spin residence (ESR) methods have been used to measure transmembrane pH gradients. Spin-labelled amines and carboxylic acids (amines and acids labelled with nitroxide free radicals) such as Tempamine and Tempacid have been used as probes to measure the pH gradient. Those probes are freely permeable to the membranes and the relative concentration of the probes within the vesicles provided a direct measurement of the pH gradient. ESR spectroscopy monitors probe partitioning between the aqueous and membrane phases giving easily resolvable signals. The effectiveness of the spin labelled nitroxide probes for determining transmembrane pH gradients has been well documented in both bacterial and animal systems. (Mehlhorn, R. and I. Probst, *Meth. Enzymol.,* 88:334-344 (1982) and Melandri, B., R. Mahlhorn, and L. Packer, "Light-Induce Proton Gradients and Internal Volumes and Chromaphores of Rhodopseudomonas Spheaeroides", *Arch. Biochem. Biophys.,* 235:97-105 (1984). However, in these previous studies these pH responsive molecules (spin labelled amines and weak acids) were used only as probes. Since these studies involved the determination of transmembrane pH gradients only very low concentrations of the pH-responsive molecules could be used so as to avoid disturbing the pH gradient being studied which was generated as a result of natural processes, e.g., the so-called proton-motive force in mitochondrial respiration.

[0023] Before going into a more detailed-explanation of the invention it will be useful to define some of the terms which are used herein. The term "lipid-like" is used broadly and includes oligolamellar lipid vesicles (liposomes), ufasomes and the like. The term chemical species having one or more selected acid or basic pH responsive groups is also used broadly to indicate any chemical or drug having acid or basic groups, properties or functions such as, but not limited to amine or carboxyl groups. Other substances such as imidazoles and barbituric acid derivatives may also be used. The term also includes any chemical that has desired chemical or therapeutic properties that will not be sufficiently altered by the attachment of such pH responsive groups. The terms "hydrophobic positively and negatively charged ions" include delocalized (i.e. membrane-permeable) cations and anions that are designated as hydrophobic ions in the literature and are ions that are capable of transmembrane migration in their charged form. The terms chemical species and "drugs" include but are not limited to, such substances as chemicals, drugs for chemotherapy and immunosuppression, membrane permeable peptide toxins and hormones. Examples of drugs having molecules having basic properties are vincristine, doxorubicin, streptomycin, chloroquine and daunorubicin. Examples of drugs having molecules having acidic properties are derivatives of methotrexate, daunomycin, penicillin, p-amino salicylic acid and salicylic acid derivatives. Examples of drugs having hydrophobic ions are ellipticinium chloride, the antihelminthics, gentian violet and pyrvinium, pamoate and other cyanine dyes and the antimalarial drug pamaguine.

[0024] The preferred vehicle for delivering drugs or chemicals to an animal in vivo are liposomes but other lipid-like vesicles may also be used. General liposomal preparation has been fully disclosed in the literature (e.g. Miyamoto, V. K. and W. Stoeckenius, "Preparation and Characteristics of Lipid Vesicles,"*J. Membrane Biol.,* 4:252-269 (1971) and U.S. Pat. No. 4,053,585). A simple highly effective preferred method for preparing vesicles is to stir soybean phosphatides (Asolectin, from Associated Concentrates) at room temperature for one-hour in either acidic or alkaline buffer and then to briefly sonicate this solution of lipids (approximately one minute). This procedure makes vesicles having large volumes of about 10 to 15 percent of the total aqueous volume within the vesicle.

[0025] The method and the kits utilize a preimposed pH gradient between the buffer in the vesicles and the solution containing the vesicles to cause the desired chemical or drug to be accumulated and encapsulated by the vesicles. Th general rule is that for every unit of pH difference a tenfold accumulation of the chemical occurs. For drugs containing several titratable groups the accumulation behavior is altered. Thus a drug which has two amino groups, having pKa's that are greater than the pH of the final solution, can be accumulated a hundred-fold with a pH gradient of one

unit. A drug with three such amino groups can be accumulated a thousand-fold in the presence of a one-unit pH gradient, etc. Conversely for a multi-acid drug, its pKa must be less than the pH of the final solution, for such substantial accumulation to occur.

[0026] The cbemicals or drugs that say be incorporated using the present method of encapsulation include those species that have acid or basic pH responsive groups, hydrophobic delocalized charged ions or that Ray be provided with such. The vesicle is prepared by the entrapment of a buffer which will not permeate the membrane in the preparation of the vesicle. The buffer is selected so as to establish the pH gradient required to take up the specific chemical species or drug. The preparation of the vesicle is carried out by stirring and sonication. If the vesicles are to be administered, parenternally, in the solution that provides the external portion of the pH gradient, they are prepared in a buffer that is either more acidic or more alkaline than the physiological pH that they will encounter in the animal.

[0027] Suhsequently the vesicles are treated with an alkaline or acid huffer, respectively, which will not permeate the vesicles memhrane, therehy causing a pH change on the exterior but not interior of the vesicles. The resulting vesicles will therefore have a pH gradient between their interior and exterior. This gradient provides the driving force for accumulating the drug or chemical within th vesicle interior. As stated before, the larger the pH gradient, the larger the concentration gradient of the drug or chemical. Although a gradient of any magnitude will accumulate a drug, considerations of directing the drug to specific tissues, while minimizing its effects on non-targeted tissues dictate that the pH gradients be maximized.

[0028] The practical limits of the pH gradients are set by the tolerance of lipid-like material that is used in preparing the vesicles. For simple biological lipids like soybean phosphatides pH extremes of 4 and about 10.5 are readily tolerated for extended periods of time. The actual pH limits for a particular preparation of vesicles could be significantly larger, depending on how long the vesicles are to be stored which in turn depends on the stability of their lipid-like constitutents. For example, vesicles to be loaded with amines are prepared in the presence of an acidic buffer such as citrate that has a pKa in the range of interest (usually about 5) and a pH of 4. This treatment ensures that the buffer will he contained within the liposome. Similarly, in cases where the liposomes are to be loaded with acidic molecules (carhoxyl groups), the liposomes are prepared by sonication in the presence of a impermeable alkaline buffer that has a pKa of ahout 10.

[0029] Examples of appropriate acidic buffers other than citrate are tartrate or succinate. Appropriate alkaline butter include besides carbonate lysine, lysine/phosphate and TAPS (ohtainable from SIGMA). The buffer may not be permeable to tbe membrane therefore buffers such as TRIS say not be used. In addition the buffer should be chloride free since chloride promotes gradient decay (the effect of physiological chloride on decay is minimal).

[0030] After tbe vesicle has been prepared, the pH of tbe solution containing the vesicle is usually adjusted by the addition of an acid or a base to a pH of, respectively, at least about 0.5, 0.3 or 0.2 pH units higher than the pH of the buffer if the buffer is acidic and the cbemical species bas respectively one, two or three remove basic pH responsive groups and at least about 0.5, 0.3 or 0.2 pH units lower tban the pH of tbe buffer if tbe buffer is basic and the cbemical species has respectively one, two or three or more acid pH responsive groups. In instances where it is desirable to inject the animal immediately with the vesicle containing solution having the adjusted pH, the pH is adjusted to a physiologically benign value of between about 7 and about 7.8, preferably about 7.4. This adjustment of the pH hy addition of an acid or hase establishes a pH gradient that drives the weak acid or base (i.e., the chemical species), into the vesicle interior. The chemical's loading rate will depend on the pKa and will be complete within less than a minute for low molecular weight (MW less than 500) amine chemicals with pKas less than 10 and having no charge or strongly polar groups other than the amino group. Analogously, weak acids having pKas greater than 4 will accumulate in the liposomes in ahout one minute, unless they hear strongly polar groups other than tbeir carboxyls. For simple &mine chemicals having a pKa greater than 11 equilibration will he slower than one minute. Analogously, a simple weak acid having a pKa lower than 4 will require more than one minute for equilibration. For more polar compounds, equilihration rates have to be determined for the specific chemicals.

[0031] The membrane of the vesicle is impermeahle to the passage of the huffer molecules throughout a ps range of 3-11. The same membrane is permeable to a chemical species which has a pKa greater than about 4 pKa units, generally 5-7 pKa units, hecause of the significant chemical potential driving the species across the membrane.

[0032] Chemicals which do not contain amino groups or equivalent basic groups or carboxyl or equivalent acid groups are first converted to a derivative containing either an acid or a base moiety that will not seriously reduce the drug's therapeutic effect. In some instances it is desirahle to prepare pro-drug moieties which will be converted into their desired active species by intracellular enzymes. Converting methotrexate to its monoester derivative as described in Example 4 is an example of such a pro-drug.

[0033] After incorporation the chemical will remain in the vesicle for fifteen minutes to several hours depending on the chemicals, until the buffer leaks out of the vesicle. One should be aware that decay of the initial drug content may occur because of dilution of the water volume outside of the vesicles when they are injected into an animal. This decay F generally occur such more slowly than the initial loading process because of favorable effects of the pH gradient on the vectorial movement of the drug across tbe vesicle membrane. This insures that a drug will reach its targeted tissue before significant leakage out of the vesicles can occur. This time period of usually several hours allows the chemical or drug to be carried to its desired destination and prevents it from acting in areas that would be deleterious to the animal.

[0034] This technique of incorporating a chemical species within a lipid-like vesicle containing a preselected buffer by means of a pH gradient can be used to rescue clinical patients who have received toxic overdoses of drugs baving acid or basic pH responsive groups (amino or carboxyl functions, etc.). Such drugs include a host of molecules sucb as general anesthetics, barbiturates (weak acids), aspirin, and other salicylates (acids) for which antidotes are not

available. Injections of large volumes of the lipsomes suspended in a solution having a physiologically benign pH (usually about 7.4) can divert the drugs from their normal biological targets such as nerve calls to the liver where they will be metabolized and henced detoxified. For some drugs like aspiring where elimation from the body does not involve significant liver metabolism, liposome injection would nevertheless provide a means for diminishing the toxic effect of the drug by reducing high blood concentrations during the initial phase of intoxication. The liposomes containing the toxin say also be removed by means of dialysis.

[0035] The kits, as described above, also utilizes a pH gradient to load lipid-like vesicles. Referring now to **FIG. 1**, it will be noted that the kit apparatus illustrated comprises a syringe **(10)** having a glass, plastic, etc. barrel **(9)** having a first compartment **(12)**, having a first solution **(13)** and a second compartment **(14)** having a second solution **(15)**. The first compartment **(12)** is separate from the second compartment **(14)** by an impermeable barrier **(16)** made of rubber, plastic or the like. The syringe **(10)** also comprises a plunger **(18)** and a needle **(20)**. The needle is surrounded by a protective sheath **(21)**. The first solution **(13)** contains the membranous vesicles **(22)** magnified in size in **FIG. 1** so as to be visible, containing a buffer **(24)** having either an acid or alkaline pH. In most instances the buffer **(24)** and the first solution **(13)** will be identical with the vesicles **(22)** having been prepared in the first solution **(13)**.

[0036] The syringe **(10)** also contains the chemical **(11)** to be loaded. The chemical **(11)** is present in the solution which affords it the greatest stability and may therefore be in either the first or second solution **(13, 15)** depending upon the properties of the chemical and the solutions. For purposes of illustration, the chemical **(11)** in **FIG. 1** is located in the first solution **(13)**. The second solution **(15)** is an acid if the buffer **(24)** inside the vesicles **(22)** is a base and a base if the buffer **(24)** inside the vesicles **(22)** is acidic. The second solution **(15)** has a pH such that a mixture of said first and second solutions **(13, 15)** will have a pH, respectively, of at least about 0.5, 0.3 or 0.2 pH units higher than the buffer **(24)** if the buffer **(24)** is acidic and the chemical **(11)** has respectively one, two or three or more basic pH responsive groups and at least about 0.5, 0.3, or 0.2 pH units lower than the pH of the buffer **(24)** if the buffer **(24)** is basic and the chemical **(11)** has respectively, on, two or three or more acid pH responsive groups. To encapsulate the chemical **(11)** within the vesicles **(22)**, the syringe **(10)** is turned with the needle **(20)** facing upwards (the opposite direction from **FIG. 1**) and the plunger **(18)** is forced upward just enough to create enough pressure to break the barrier **(16)**. The barrier may also be broken by a sharp implement **(19)** attached to the plunger **(18)**. In this instance the implement **(19)** is prevented from damaging the barrel **(9)** by a stop **(23)**. The volume of the second solution **(15)** in the second compartment **(14)** should be such that when the barrier **(16)** is broken only a minimal amount of solution will enter the needle **(20)**. Once the barrier **(16)** is broken the plunger **(18)** is withdrawn to around its initial position at the top of the first compartment **(12)**. The syringe **(10)** is then agitated and the two solutions are allowed to mix for an appropriate period of time. If the resulting pH of the mixture of the first and second solutions **(13, 15)** is physiologically benign the entire mixture including the vesicles **(22)** containing the now encapsulated chemical species may be injected directly into an animal. For facilitation in operating the plunger **(18)** the syringe may have a block **(26)** at the bottom of the barrel **(9)** with an air hole **(28)**.

[0037] **FIG. 2** illustrates another embodiment of the invention. This embodiment in better understood by way of reference to **FIG. 1** of the drawings whereas like numbers in **FIGS. 1 and 2** refer to like parts. In this instance, the barrel **(9)** has only one compartment **(12)** containing a solution **(13)** having vesicles **(22)** containing the buffer **(24)**. To encapsulate the chemical **(11)** which Lay or may not be included with the kit but which for purposes this illustration is included in the first solution **(13)**, the contents of the syringe **(10)** are emptied into a first vial **(30)**. The first vial **(30)** may be composed of glass, plastic or the like. The contents of the barrel **(9)** are emptied into the first vial **(30)** by piercing the rubber septum **(32)** with the needle **(20)** and pushing the plunger **(18)** downward. The rubber septum **(32)** is kept in place by a retaining clip **(34)**. The first vial **(30)** also contains a second solution **(15)** which is an acid if the buffer **(24)** is a base and a base if the buffer **(24)** is acidic. The second solution **(15)** has a pH such that mixture of said first and second solutions **(13, 15)** will have a pH respectively of at least about 0.5, 0.3 or 0.2 pH units higher than the buffer **(24)** if the buffer **(24)** is acidic and the chemical **(11)** has respectively one, two, or three or more basic pH responsive groups and at least about 0.5, 0.3 or 0.2 pH units lover than the pH of the buffer **(24)** if the buffer **(24)** is basic and the chemical species has respectively, one, two, or three or more acid pH responsive groups. The first solution **(13)** and the second solution **(15)** are allowed to mix to encapsulate the chemical **(11)** and the syringe **(10)** is refilled and emptied several times. The procedure described above is repeated for a second vial **(34)**. The second vial contains a third solution **(36)** which is acid if the combination of the first and second solution **(13, 15)** is basic and basic if the combination is acid. In addition the pH of the third solution **(36)** has been calculated so as to render the mixture of all three solutions physiologically benign with regard to the blood of a mammal.

EXAMPLE 1

[0038] Liposomes of soybean lipids were prepared according to a variation of Miyamoto and Stoeckenius, supra by sonication of 1 gm of asolectin in the presence of 10 mls of 100 mM sodium citrate at pH 5.0. Spin-labeled primary amine Tempamine (Aldrich Chemical Co.) was added to 50 μM citrate solution containing the pre-sonicated vesicles to give a final concentration of 20 μM, and a sufficient amount of 5 molar sodium hydroxide was also added to the solution to raise the pH of the solution to 7.4. This resulted in a 300-fold accumulation of the Tempamine inside the vesicles within one minute of the addition of the base. The rate of uptake of the amino depends on the pKa of the amino. As determined by ESR spectroscopy the resulting pH gradient was stable for several hours.

EXAMPLE 2

[0039] Liposomes were prepared by sonicating 0.5 grams of asolectin in 10 mls of 100 mM sodium citrate buffer, pH 4. An amount of 542 microliters of five normal sodium hydroxide was added. This raised the pH of the bulk solution containing the liposomes to 7.4. An intravenous catheter system consisting of a 27-gauge needle, connected to a 1.0

ml syringe by 4 inches of PE20 (polyethylene) plastic tubing was used for the infusion of the liposome suspension into the lateral tail veins of two female Wistar rats, 250 grams each. The liposome suspension was infused into the rats at a rate of about 0.2 mls per minute until a total volume of 0.7 mls had been infused. The rats appeared somewhat disoriented upon completion of the infusion, and release from the restraining cones, but otherwise none the worse from the experience. One hour later the animals were examined and were completely normal in appearance, and after one week's observation, no long-term effects of the infusion could be detected.

EXAMPLE 3

[0040] Lipid vesicles, containing 15 mg/ml of Sigma Type II-S phosphatidyl choline were prepared by sonication in a 120 mM lysine/phosphate buffer (chloride-free) at pH 10.5. The total sonication time was three minutes, with intermittent cooling. The vesicles were incubated for two minutes with 20 μM of a spin-labeled carboxylic acid, prepared by reacting 1M succinic anhydride with one equivalent of Tempamine in choloroform, in the presence of a sufficient amount of a 100 mM citric acid to lower the external pH to 6 (approximately 1 volume equivalent). Analysis of the intravesicular concentration of the spin-labeled acid by ESR spectroscopy revealed that a more than 1,000-fold increase had occurred in response to the imposed pH gradient.

[0041] The vesicles were then transferred into a piece of dialysis tubing that had been spread into a flattened geometry to minimize the diffusion path of internal molecules to its surface. When the dialysis tubing was placed in a large volumes of phosphate buffer in isotonic saline solution, this system simulated the physiological situation that would arise when vesicles are injected into the blood, where dilution of the drug outside the vesicles would occur as the vesicles moved through the circulation. When the tubing was placed into a beaker containing more than a ten-fold excess of lysine buffer; the pH gradient that had been preimposed was largely collapsed upon mixing of the aqueous phases inside and outside of the tubing. Table 1 shows the kinetics of efflux of the spin-labeled acid out of the dialysis tubing, and also shows the kinetics of the same probe when incubated with vesicles that have not been subjected to a pH gradient.

[0042] It is clear from the data in Table 1 that when the intradialysis concentration of probes was examined at the end of the incubation period, the vesicles that had been loaded with the pH gradient had retained a such higher concentration of the acid than those without a pH gradient. This example also indicates that it is unnecessary to maintain the pH gradient subsequent to the chemical loading procedure.

TABLE 1

ESR signal leaking out of dialysis tubing containing vesicles that had been incubated with a spin labelled carboxylic acid in the presence and absence of a pH gradient.

No pH gradient		pH gradient	
Time (min)	ESR signal	Time (min)	ESR signal
3	0.09	15	0.11
10	0.15	30	0.12
20	0.17	45	0.15
40	0.18		
internal	0.24	internal	3.0

EXAMPL 4

[0043] Methotraxate is converted to its monoester derivative by synthesizing methotrexate from the monomethyl ester of glutamate rather than from glutamate itself by conventional methods for methotrexate synthesis. Liposomes are prepared as in Example 3 and the vesicles are incubated for about ten minutes with 1 mg ml of the methotrexate derivative in the presence of a sufficient amount of 100 mM citric acid to lower the external pH to 4 (approximately one volume equivalent). The methotrexate is thereby internalized within the vesicles. The external pH is adjusted to 7.4 and 0.1 ml of the solution is injected into mice representing approximately 4% of total fluid body volume (2.5 ml).

EXAMPLE 5

[0044] Liposomes are prepared according to Example 1 or 2 and are concentrated by means of a standard filtration concentration to a concentration of approximately 50 mg asolectin per 1 ml of 100 mM sodium citrate. The resulting lipid-like solution is injected in mice as described in Example 2 such that the final infusion is approximately 1% of total fluid body volume of asolectin. This example indicates that large volumes of liposomes having substantial pH gradients can be injected into animals without serious adverse effects.

Example 6

[0045] Loading of Hydrophobic Ions:

[0046] Vesicles are prepared at pH 4.5 as before. The vesicle solution contains 10 uM of the cyanine dye dithiasanine iodide. To achieve internalization of the cyanine dye, the vesicles are mixed with a 100 mM solution of sodium triphosphate of sufficient volume to raise the pH of the mixture to 7.4. This generates a pH gradient acid-inside in the vesicles and this pH gradient in turn generates an electrical gradient of about 180 millivolts, negative inside the vesicles. The positively charged cyanine dye, whose delocalized charge renders it membrane permeable, is driven into the vesicle interior in response to the electrical potential, reaching a final accumulation of a thousand fold relative to the aqueous solution outside of the vesicles. Since the vesicles are prepared with a internal volume of bout 10%, the final cyanine concentration inside the vesicles is about 100 uM, while the external cyanine concentration is bout 100 nM.

INDUSTRIAL APPLICABILITY

[0047] In accordance with the present invention, kits and methods are provided for encapsulating any number of drugs

within lipid-like vesicles. A method for detoxifying an animal is also described. The kits are very easy to use and require little or no training for the operator. They are fast and provide a hgh rate of encapsulation. Although the foregoing invention has been described in some detail by way of illustration and example for the purposes of clarity and understanding, it sbould be recognized that changes and modifications may be practiced within the scope of the appended claims.

1. A metbod of loading a lipid-like vesicles having a membrane permeable to a cbemical species to be loaded from a loading solution wherein the concentration of the thus loaded cbemical species within the vesicle Is greater than the concentration of the chemical species in the loading solution and tbe loaded chemical species can be substantially maintained within the vesicle for at least one-quarter hour following loading, comprising:

inducing a pH gradient between the inside and outside of the vesicle membrane while the vesicle is in tbe loading solution containing the chemical species said pH gradient being selected to drive said chemical species into said vesicles.

2. A metbod of loading lipid-like vesicles having a membrane permeable to a chemical species to be loaded and for substantially maintaining the loaded chemical species within tbe vesicle for at least one-quarter hour following loading by inducing a pH gradient across the membrane, comprising:

(1) incorporating within the vesicle a buffer solution buffered to a selected acid or alkaline pH and having a selected molarity and at least one selected pKa approximately equal to the selected buffer pH, the membrane being substantially impermeable to the buffer for at least one-quarter hour following loading of the chemical species;

(2) positioning the vesicles in a bulk solution having a selected pH; and

(3) providing the bulk solution witb a chemical species having one or more selected acid pH responsive groups if the buffer is alkaline or one or' more basic pH responsive groups if the buffer is acidic wherein the pH of the bulk solution is at least respectively 0.5, 0.3 or 0.2 of a pH unit higher than the pH of the buffer if the buffer is acidic and the chemical species bas respectively one, two, or three or more basic pH responsive groups, or the pH of the bulk solution is at least respectively 0.5, 0.3 or 0.2 of a pH unit lower than the pH of the buffer if the buffer is basic and the chemical species bas respectively one, two, or three or more acid pH responsive groups, the pH responsive groups of the cbemical species having one or more, acid pH responsive groups have a pKa that is generally lower than or equal to the pH of the bulk solution and generally higber than or equal to 3.5 and the pH responsive groups of the cbemical species having one or more basic pH responsive groups have a pKa that is generally higher than or equal to the pH of the bulk solution and generally lower than or equal toll.

3. A method according to claim 2 wherein the pH responsive group or groups are acid pH responsive groups and the buffer has a pKa in the range of about 10.

4. A metbod according to claim 3 wherein the chemical species has a pKa from about 4-7.

5. A method according to claim 4 wberein the pH responsive group is a carboxyl group.

6. A method according to claim 2 wherein tbe pH responsive group or groups are basic pH responsive groups, and the buffer has a pKa in the range of about S.

7. A method according to claim 6 wherein tbe chemical species has a pKa from about 7-10.

8. A method according to claim 7 wherein the pH responsive group is an amino group.

9. A method according to claim 8 wherein tbe cbemical species is an amine.

10. A method according to claim 2 wberein tbe vesicle is prepared in the buffer and incorporates the buffer via mixing and sonication.

11. A method according to claim 2 wberein tbe pH of the bulk solution is from about 7.0 to about 7.8.

12. A method according to claim 11 wherein the pH of the bulk solution is bout 7.4.

13. Vesicles prepared according to claim 2 wherein the chemical species is a drug.

14. A pharmaceutical preparation for administration in vivo to an animal comprising lipid-like vesicles prepared according to claim 1 wherein said chemical species is a drug.

15. A pharmaceutical preparation for parenteral administration in vivo to an animal comprising liposomes prepared according to claim 2 wherein said chemical species is a drug, the osmolarity of the buffer is within the pbysiological range of the animal, the vesicles are suspended for administration in the bulk solution, and the pH of the bulk solution is physiologically benign.

16. A kit for loading lipid-like vesicles having a membrane permeable to the chemical species to be loaded comprising:

(1) a first compartment baving a first solution baving membranous lipid-like vesicles incorporating a buffer buffered to a selected acid or basic pH, the buffer having at least one selected pKa approximately equal to the selected buffer pH and a selected molarity and being substantially impermeable to the vesicle for at least one-quarter hour following loading of tbe chemical species and the first solution having a selected pH such that the stability of the vesicle and its buffer can be maintained for a period of at least one week at 4° C.

(2) a second compartment, separate from the first compartment, having a second solution having a selected pH;

(3) a chemical species permeable to the vesicle having a selected pKa and one or more selected acid pH responsive groups if tbe buffer is basic or one or more basic pH responsive groups if the buffer is acidic, the chemical species being initially present in a selected one of the two solutions with the second solution baving a ps such that a mixture of the first and second solutions would have a pH respectively of at least, 0.5, 0.3, or 0.2 of a pH unit higher than the pH of the buffer if the buffer is acidic and the chemical species bas respectively one, two, or three or more basic pH responsive groups or a pH at least, respectively, 0.5, 0.3 or 0.2 of a pH unit lower than the pH of the buffer if the buffer is basic and the chemical species has respectively one, two or three or more acid pH responsive groups, the pH responsive groups of the chemical specics having one or more acid pII responsive groups have a pKa that is

generally lower than or equal to the pH of the mixture of the first and second solution and generally higher than or equal to 3.5 and the pH responsive groups of the chemical species having one or more basic pH responsive groups have a pKa that is generally higher than or equal to the pH of the mixture of the first and second solutions and generally lower than or equal to 11.

17. A kit as set forth in claim 16 wherein said chemical species is a drug.

18. A kit an set forth in claim 17 wherein the mixture will have a pH that is physiologically benign in regard to the blood of a mammal.

19. A kit as set forth in claim 18 further including means for parenterally delivering the mixture to a mammal in vivo.

20. A kit for loading lipid-like vesicles having a membrane permeable to an acid or basic chemical species to be loaded comprising:

(1) a first compartment having a first solution having membranous lipid vesicles incorporating a buffer buffered to a selected basic pH if the chemical species to be loaded is an acid or acid pH if the species in a base, the buffer having a selected pKa and a selected molarity, the membrane being substantially impermeable to the buffer for at least one-quarter hour following loading of the chemical species, the first solution having a selected pH such that the stability of the vesicle and its buffer will be maintained for a period of at least one week at 4° C.;

(2) a second separate compartment having a first substance which when combined with the first solution will adjust the pH of the first solution so as to provide a predetermined pH gradient between the buffer within the vesicle and the pH adjusted first solution; and

(3) a third separate compartment having a second substance which when combined with the pH adjusted first solution will further change the pH of said solution to a physiologically benign value with regard to the blood of a mammal.

21. A kit as set forth in claim 20 further including a selected chemical species.

22. A kit as set forth in claim 21 wherein the selected chemical species is a drug.

23. A kit as set forth in claim 22 further including a means for parentally delivering the vesicle solution having the physiologically benign adjusted pH to a mammal in vivo.

24. A method of detoxifying an animal suffering from an overdose of a chemical species with basic pH responsive groups comprising injecting the animal with a solution having a physiologically benign pH with respect to the animal, the solution having large volumes of liposomes having a buffer solution buffered to a pH generally lower than or equal to 5.4 and the buffer having at least one selected pKa and a selected molarity within the physiological range of the animal the liposomes being substantially impermeable to the buffer for at least one hour after injection.

25. A method for detoxifying an animal suffering from an overdose of a chemical species with acid pH responsive groups the chemical species being permeable to liposomes comprising: injecting the animal with a solution having a physiologically benign pH with respect to the animal, the solution having large volumes of liposomes having a buffer solution buffered to a pH generally higher than or equal to 9.4 and having a selected solarity and selected pKa, the liposomes being substantially impermeable to the buffer for at least one hour after injection.

26. A method of loading lipid-like vesicles having a membrane permeable to a chemical species to be loaded and substantially maintaining the loaded chemical species by inducing a pH gradient across the membrane within the vesicle for at least one-quarter hour following loading, comprising:

(1) incorporating within the vesicle a buffer solution buffered to a selected acid or alkaline pH and having a selected molarity and at least one selected pKa, the membrane being substantially impermeable to the buffer for at least one-quarter hour following loading of the chemical species;

(2) positioning the vesicles in a bulk solution having a selected pH of either 0.5 to 3 pH units lower or pH units higher than the pH of the buffer thereby establishing a transmembrane electrical potential and a positive charge inside the vesicle if the pH outside the vesicle is more acid than inside or a negative charge inside the cell if the pH outside the call is more basic than inside;

(3) providing in the bulk solution a chemical species having hydrophobic negatively-charged ions if the membrane charge within the vesicle is positive or hydrophobic positively charged ions if the membrane charge within the vesicle is negative.

* * * * *

EXHIBIT G

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:	
MEHLHORN, Rolf Joachim	**Group Art Unit:** 1614
Serial No.: 10/759,222	**Examiner:** Weddington, Kevin E.
Filed: 20 January 2004	**Confirmation No.:** 6448
For: METHODS FOR LOADING LIPID LIKE VESICLES WITH DRUGS OR OTHER CHEMICALS	

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RESPONSE TO NON-FINAL OFFICE ACTION

With regard to the non-final office action mailed 29 November 2007, please take the following actions and consider the remarks below.

IN THE SPECIFICATION

Please amend the specification as shown on the following separate pages.

IN THE CLAIMS

Please amend the claims as shown on the separate pages following the amendments to the specification.

AMENDMENTS TO THE SPECIFICATION

Please replace the sentences of the indicate paragraphs with the corrected versions shown below:

1. Paragraph **[0010]**, last sentence:

The pH of the bulk solution is at least ~~respectively~~ 0.5, 0.3, or 0.2 [[f]] of a pH unit lower than [[th]] the pH of the buffer of the buffer is basic and [[th]] the chemical species has respectively one, two or three or more acid responsive groups.

2. Paragraph **[0018]**, sentence beginning on line 19:

Drugs that have ~~deloterious~~ deleterious general effects such as chemotherapeutic or immuno-suppressant drugs may be encapsulated in this manner and used to treat specific tissues or ~~calls~~ cells.

3. Paragraph **[0018]**, sentence beginning at line 22:

Because of the high rate of encapsulation and the efficiency of encapsulation, concern over the expense and scarcity of ~~the~~ chemotherapeutic drugs no longer need be as great since only insignificant quantities of the drug will ~~rein~~ remain in the loading solution following vesicle preparation.

4. Paragraph **[0026]**, first sentence:

The chemicals or drugs that ~~say~~ may be incorporated using the present method of encapsulation include those species that have acid or basic pH responsive groups, hydrophobic delocalized charged ions or that ~~Ray~~ may be provided with such.

4. Paragraph **[0029]**, second sentence:

Appropriate alkaline ~~butter~~ buffers include besides ~~carbonate~~ lysine/carbonate, lysine/phosphate and TAPS (obtainable from SIGMA).

5. Paragraph **[0030]**, first sentence:

After the vesicle has been prepared, the pH of the solution containing the vesicle is usually adjusted by the addition of an acid or base to a pH ~~of, respectively,~~ at least about 0.5, 0.3 or 0.2 pH units higher than the pH of the buffer if the buffer is acidic and the chemical species has ~~respectively~~ one, two or three ~~remove~~ basic pH responsive groups and at least about 0.5, 0.3 or 0.2 pH units lower than the pH of the buffer if the buffer is basic and the chemical species has ~~respectively~~ one, two or three or more acid pH responsive groups.

6. Paragraph **[0030]**, second to last sentence:

For simple ~~&mine chemicals~~ amines having a pKa greater than 11, equilibration will be slower than one minute.

7. Paragraph **[0031]**, first sentence:

The membrane of the vesicle is impermeable to the passage of the buffer molecules throughout a [[ps]] pH range of 3-11.

8. Paragraph **[0033]**, third sentence:

This decay [[F]] will generally occur ~~such~~ much more slowly than the initial loading process because of favorable effects of the pH gradient on the vectorial movement of the drug across the vesicle membrane.

9. Paragraph **[0034]**, fourth sentence:

For some drugs like ~~aspiring~~ aspirin where ~~elimation~~ elimination from the body does not involve significant liver metabolism, liposome injection would nevertheless provide a means of diminishing the toxic effect of the drug by reducing high blood concentrations during the initial phase of intoxication.

10. Paragraph **[0034]**, last sentence:

The liposomes containing the toxin ~~say~~ may also be removed by means of dialysis.

11. Paragraph **[0035]**, third sentence:

The first compartment **(12)** is ~~separate~~ separated from the second compartment **(14)** by an impermeable barrier **(16)** made of rubber, plastic or the like.

12. Paragraph **[0036]**, 5th sentence:

The second solution **(15)** has a pH such that a mixture of said first and second solutions **(13, 15)** will have a pH~~, respectively,~~ of at least about 0.5, 0.3 or 0.2 pH units higher than the buffer **(24)** if the buffer **(24)** is acidic and the chemical **(11)** has ~~respectively~~ one, two, [[or]] three or more basic pH responsive groups and at least about 0.5, 0.3, or 0.2 units lower than the pH of the buffer **(24)** if the buffer **(24)** is basic and the chemical **(11)** has ~~respectively, on~~ one, two, [[or]] three or more acid pH responsive groups.

13. Paragraph **[0037]**, 4th sentence:

To encapsulate the chemical **(11)** which ~~Lay~~ may or may not be included with the kit but which ~~for purposes~~ for the purpose of this illustration is included in the first solution **(13)**, the contents of the syringe **(10)** are emptied into a first vial **(30)**.

14. Example 1, last two sentence:

The rate of uptake of the ~~amino~~ amine depends on the pKa of the ~~amine~~ amine._As determined by ESR spectroscopy the resulting pH gradient was stable for several hours.

15. Example 6, last sentence:

Since the vesicles are prepared with [[a]] an internal volume of ~~bout~~ about 10%, the final cyanine concentration inside the vesicles is about 100 μM, with the external cyanine concentration is ~~bout~~ about 100 nM.

AMENDED CLAIM SET

1. (CURRENTLY AMENDED) A method of loading [[a]] lipid-like vesicles ~~having a membrane permeable to a~~ with a chemical species ~~to be loaded from a loading solution wherein the concentration of the thus loaded chemical species within the vesicle [[Is[[is greater than the concentration of the chemical species in the loading solution and the loaded chemical species can be substantially maintained within the vesicle for at least one-quarter hour following loading~~, comprising:

forming lipid-like vesicles in a solution comprising an acidic buffer if the chemical species to be loaded is basic or a basic buffer if the chemical species to be loaded is acidic; wherein:

membranes of the formed lipid-like vesicles are impermeable to the buffer;

adjusting the pH of the solution exterior to the membranes of the lipid-like vesicles to a basic pH if the chemical species to be loaded is basic or to an acidic pH if the chemical species to be loaded is acidic;

adding a basic chemical species to the adjusted basic exterior solution or an acidic chemical species to the adjusted acidic exterior solution;

loading the chemical species into the vesicle; and

adjusting the exterior solution to a physiologically benign pH; wherein:

the chemical species is substantially maintained in the vesicle for at least one quarter hour after the adjustment of the exterior solution.

2. (CURRENTLY AMENDED) A method of loading lipid-like vesicles having a membrane permeable to a chemical species to be loaded and for substantially maintaining the loaded chemical species within the vesicle for at least one-quarter hour following loading by inducing a pH gradient across the membrane, comprising:

(1) incorporating within the vesicle a buffer solution buffered to a selected acid or alkaline pH having a selected molarity and at least one selected pKa approximately equal to the selected buffer pH, the membrane being substantially impermeable to the buffer for at least one-quarter hour following loading of the chemical species;

(2) positioning the vesicles in a bulk solution having a selected pH; and
(3) providing the bulk solution with a chemical species having one or more selected acid pH responsive groups if the buffer is alkaline or one or more basic pH responsive groups if the buffer is acidic wherein the pH of the bulk solution is at least ~~respectively~~ 0.5, 0.3 or 0.2 of a pH unit higher than the pH of the buffer if the buffer is acidic and the chemical species has ~~respectively~~ one, two, or three or more basic pH responsive groups, or the pH of the bulk solution is at least ~~respectively~~ 0.5, 0.3 or 0.2 of a pH unit lower than the pH of the buffer if the buffer is basic and the chemical species has ~~respectively~~ one, two or three or more acid pH responsive groups, the pH responsive groups of the chemical species having one or more acid pH responsive groups have a pKa that is generally lower than or equal to the pH of the bulk solution and generally higher than or equal to 3.5 and the pH responsive groups of the chemical species having one or more basic pH responsive groups have a pKa that is generally higher than or equal to the pH of the bulk solution and generally lower than or equal ~~toll~~ <u>to 11</u>.

3. (CURRENTLY AMENDED) A method according to claim 2 wherein the pH responsive group or groups are acid pH responsive groups and the buffer has a pKa ~~in the range~~ of about 10.

4. (CURRENTLY AMENDED) A method according to claim 3 wherein the chemical species has a pKa ~~from~~ <u>of</u> about 4-7.

5. (PREVIOUSLY PRESENTED) A method according to claim 4 wherein the pH responsive group is a carboxyl group.

6. (CURRENTLY AMENDED) A method according to claim 2 wherein the pH responsive group or groups are basic pH responsive groups, and the buffer has a pKa in the range of about [[S]] <u>5</u>.

7. (PREVIOUSLY PRESENTED) A method according to claim 6 wherein the chemical species has a pKa from about 7-10.

8. (PREVIOUSLY PRESENTED) A method according to claim 7 wherein the pH responsive group is an amino group.

9. (PREVIOUSLY PRESENTED) A method according to claim 8 wherein the chemical species is an amine.

10. (PREVIOUSLY PRESENTED) A method according to claim 2 wherein the vesicle is prepared in the buffer and incorporates the buffer via mixing and sonication.

11. (CURRENTLY AMENDED) A method according to claim 2 wherein the pH of the bulk solution is ~~from~~ about 7.0 to about 7.8.

12. (CURRENTLY AMENDED) A method according to claim 11 wherein the pH of the bulk solution is ~~bout~~ <u>about</u> 7.4.

13. (WITHDRAWN AND CURRENTLY AMENDED) ~~Vesicles prepared according to~~ <u>The method of</u> claim 2 wherein the chemical species is a drug.

14. (WITHDRAWN) A pharmaceutical preparation for administration in vivo to an animal comprising lipid-like vesicles prepared according to claim 1 wherein said chemical species is a drug.

15. (WITHDRAWN AND CURRENTLY AMENDED) A pharmaceutical preparation for parenteral administration in vivo to an animal comprising ~~liposomes~~ <u>lipid-like vesicles</u> prepared according to claim 2 wherein said chemical species is a drug, the osmolarity of the buffer is within the physiological range of the animal, the vesicles are

suspended for administration in the bulk solution, and the pH of the bulk solution is physiologically benign.

16. (CURRENTLY AMENDED) A kit for loading lipid-like vesicles having a membrane permeable to the chemical species to be loaded comprising:

(1) a first compartment having a first solution having membranous lipid-like vesicles incorporating a buffer buffered to a selected acid or basic pH, the buffer having at least one selected pKa approximately equal to the selected buffer pH and a selected molarity and being substantially impermeable to the vesicle's membrane for at least one-quarter hour following loading of the chemical species and the first solution having a selected pH such that the stability of the vesicle and its buffer can be maintained for a period of at least one week at ~~4° C~~ 4 °C.

(2) a second compartment, separate from the first compartment, having a second solution having a selected pH;

(3) a chemical species permeable to the vesicle having a selected pKa and one or more selected acid pH responsive groups if the buffer is basic or one or more basic pH responsive groups if the buffer is acidic, the chemical species being initially present in a selected one of two solutions with the second solution having a [[ps]] pH such that a mixture of the first and second solutions would have a pH ~~respectively of~~ at least 0.5, 0.3, or 0.2 of a pH unit higher than the pH of the buffer if the buffer is acidic and the chemical species has ~~respectively~~ one, two, or three or more basic pH responsive groups ~~or a pH~~ at least~~, respectively,~~ 0.5, 0.3 or 0.2 of a pH unit lower than the pH of the buffer if the buffer is basic and the chemical species has ~~respectively~~ one, two or three or more acid pH responsive groups, the pH responsive groups of the chemical species having one or more acid pH responsive groups have a pKa that is generally lower than or equal to the pH of the mixture of the first and second solution and generally higher than or equal to 3.5 and the pH responsive groups of the chemical species having one or more basic pH responsive groups have a pKa that is generally higher than or equal to the pH of the mixture of the first and second solutions and generally lower than or equal to 11.

17. (PREVIOUSLY PRESENTED) A kit as set forth in claim 16 wherein said chemical species is a drug.

18. (CURRENTLY AMENDED) A kit [[an]] as set forth in claim 17 wherein the mixture will have a pH that is physiologically benign in regard to the blood of a mammal.

19. (CURRENTLY AMENDED) A kit as set forth in claim 18 further ~~including~~ comprising means for parenterally delivering the mixture to a mammal in vivo.

20. (CURRENTLY AMENDED) A kit for loading lipid-like vesicles having a membrane permeable to an acid or basic chemical species to be loaded comprising:

(1) a first compartment having a first solution having membranous ~~lipid~~ lipid-like vesicles incorporating a buffer buffered to a selected basic pH if the chemical species to be loaded is an acid or acid pH if the species in a base, the buffer having a selected pKa and a selected molarity, the membrane being substantially impermeable to the buffer for at least one-quarter hour following loading of the chemical species, the first solution having a selected pH such that the stability of the vesicle and its buffer will be maintained for a period of at least one week at [[4° C.]] 4 °C;

(2) a second separate compartment having a first substance which when combined with the first solution will adjust the pH of the first solution so as to provide a predetermined pH gradient between the buffer within the vesicle and the pH adjusted first solution; and

(3) a third separate compartment having a second substance which when combined with the pH adjusted first solution will further change the pH of said solution to a physiologically benign value with regard to the blood of a mammal.

21. (CURRENTLY AMENDED) A kit as set forth in claim 20 further ~~including~~ comprising a selected chemical species.

22. (PREVIOUSLY PRESENTED) A kit as set forth in claim 21 wherein the selected chemical species is a drug.

23. (PREVIOUSLY PRESENTED) A kit as set forth in claim 22 further including a means for parentally delivering the vesicle solution having the physiologically benign adjusted pH to a mammal in vivo.

24. (WITHDRAWN) A method of detoxifying an animal suffering from an overdose of a chemical species with basic pH responsive groups comprising injecting the animal with a solution having a physiologically benign pH with respect to the animal, the solution having large volumes of liposomes having a buffer solution buffered to a pH generally lower than or equal to 5.4 and the buffer having at least one selected pKa and a selected molarity within the physiological range of the animal the liposomes being substantially impermeable to the buffer for at least one hour after injection.

25. (WITHDRAWN) A method for detoxifying an animal suffering from an overdose of a chemical species with acid pH responsive groups the chemical species being permeable to liposomes comprising: injecting the animal with a solution having a physiologically benign pH with respect to the animal, the solution having large volumes of liposomes having a buffer solution buffered to a pH generally higher than or equal to 9.4 and having a selected solarity and selected pKa, the liposomes being substantially impermeable to the buffer for at least one hour after injection.

26. (WITHDRAWN) A method of loading lipid-like vesicles having a membrane permeable to a chemical species to be loaded and substantially maintaining the loaded chemical species by inducing a pH gradient across the membrane within the vesicle for at least one-quarter hour following loading, comprising:

(1) incorporating within the vesicle a buffer solution buffered to a selected acid or alkaline pH and having a selected molarity and at least one selected pKa, the

membrane being substantially impermeable to the buffer for at least one-quarter hour following loading of the chemical species;

(2) positioning the vesicles in a bulk solution having a selected pH of either 0.5 to 3 pH units lower or pH units higher than the pH of the buffer thereby establishing a transmembrane electrical potential and a positive charge inside the vesicle if the pH outside the vesicle is more acid than inside or a negative charge inside the cell if the pH outside the call is more basic than inside;

(3) providing in the bulk solution a chemical species having hydrophobic negatively-charged ions if the membrane charge within the vesicle is positive or hydrophobic positively charged ions if the membrane charge within the vesicle is negative.

REMARKS

Claims 1-26 are currently before the examiner. Claims 13-15 and 24-26 have been withdrawn by the examiner as, in the examiner's view, being drawn to separate and distinct invention. Applicant traversed the withdrawal, which the examiner did not deem persuasive. Claims 1-12 and 16-23 stand rejected. Claims 1-4, 6, 11, 12, 16, and 18-21 have been amended. The amendments are made either to correct typographic errors or to more accurately describe that which the inventor considers the invention. All amendments are fully supported by the specification; no new matter has been added.

Objection to the specification

The examiner has objected to the specification as containing a number of misspelled words and a number of words with letters missing and has required correction of same.

Applicant's response

Applicant has amended the specification to correct all such errors that applicant could find at this time.

Double patenting

The examiner has issued a non-statutory double patenting rejection of claims 16-23 over claims 1-7 of U.S. Pat. No. 5,762,957.

Applicant traverses.

Applicant's response

While the examiner notes that the present claims include an "additional step" applied to the kit claimed therein and then speaks in terms of the present claims "anticipating the patented claims because the patented claims recite 'comprising' and thus opens the claims to the inclusion of additional steps," it is understood that a non-statutory terminal disclaimer requires determination of whether the challenged claims

are an obvious variation of an invention claimed in the patent. Applicant respectfully submits that they are not.

The kits of the current claims require a first compartment containing an acidic or basic buffer solution both within and outside lipid-like vesicles, wherein the vesicles are substantially impermeable to the buffer for at least one-quarter hour following loading of the chemical species and, further, the first solution has a pH selected such that the stability of the vesicle and its buffer can be maintained for a period of at least one week at 4 °C. The current claims also require a second compartment having a second solution at a selected pH wherein when the first and second solutions are mixed the resulting solution has a pH that is 0.5, 0.3 or 0.2 pH units above the pH of the buffer in the liposomes if the buffer is acidic or 0.5, 0.3 or 0.2 pH units below the pH of the buffer in the liposomes if the buffer is basic.

The patented kits, on the other hand, make no mention whatsoever as to the duration of the impermeability of its liposome membranes to the buffer or to the selection of a first solution pH so as to result in stable vesicles and buffers for at least one week. Further, the patented invention says nothing about the pH of the combined first and second solutions. Thus the patented kit requires only acids and bases and does not specify any particular pH while the current application does. The difference, i.e., from no preference to the express limitations of the current invention are not obvious.

The examiner is requested to reconsider and thereupon withdraw the terminal disclaimer over U.S. Pat. No. 5,762,957.

Double patenting over U.S. Pat. No 5,827,532.

The examiner has also issued a non-statutory double patenting rejection of claims 1-12 over claims 1-9 of U.S. Pat. No. 5,827,532.

Applicant's response

Submitted herewith is a terminal disclaimer disclaiming the term of a patent that issues from this application that exceeds the maximum permitted term of the '532 patent.

35 U.S.C. § 112, second paragraph, rejection of claims 1-12 and 16-23

The examiner has rejected claims 1-12 and 16-23 under § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter that application regards as the invention. In particular, the examiner points out a number of errors and words missing in the claims.

Applicant's response

Applicant has amended the claims to, among other things, correct the scrivener's errors therein.

35 U.S.C. § 102 rejection of claim 1

The examiner has rejected claim 1 over § 102(b) as being anticipated by Nichols, et al., *Biochemica et Biophysica Acta*, **1976**, 455:269-7. In the examiner's opinion, Nichols teaches a method of preparation of liposomes using the method set forth in claim 1. That is, as the examiner sees it, the method involved preparing liposomes with acid pH and titrating them with a base to create a pH gradient and adding a basic drug, which include epinephrine, to load the drug.

Applicant traverses.

Applicant's response

Claim 1 has been amended to more fully assert that which the inventor considers the invention. As such the above rejection is rendered moot. That is, Nichols is concerned only with the uptake of an amine, catechol amine, epinephrine, norepinephrine or dopamine, into an liposome using a pH gradient. Nichols does not mention, in fact clearly is not in the least concerned with, how long the amine remains in the liposome after the gradient is destroyed as clearly the case in amended claim 1 since no data or discussion of such appears in the paper.

The examiner is requested to reconsider and thereupon withdraw the rejection.

35 U.S.C. § 102 rejection of claim 1

The examiner has also rejected claim 1 under §102(b) as being anticipated by Deamer, et al., *Biochemica et Biophysica Acta*, **1972**, 274:323-335. In the examiner's opinion, Deamer teaches a method of preparation of liposomes using the method of claim 1. That is, the examiner notes that Deamer loads amines by preparing liposomes with acid pH and titrating them with a base to create a pH gradient and adding a basic amine.

Applicant traverses.

Applicant's response

As was the case with Nichols, applicants' amendment to claim 1 renders the rejection over Deamer moot. Deamer is concerned solely with the development of a probe for measuring the actual pH gradient across membranes. At no point does Deamer discuss or even allude what happens when the pH gradient is destroyed when a membrane impermeable buffer is used inside the membrane or, more specifically, to containing an absorbed chemical species within a liposome for a period of time after such destruction of the membrane.

The examiner is requested to reconsider and thereupon withdraw the rejection.

35 U.S.C. §102 rejection of claim 1

The examiner again rejects claim 1 under §102(b) as being anticipated by Cramer, et al., *Biochem and Biophys Research Communications*, **1977**, 75(2):295-301. In the examiner's view, Cramer teachs the method of claim 1 in it involves the preparation of liposomes and then the lowering of the pH or the external medium so as to load acidic compounds.

Applicant traverses.

Applicant's response

Like Nichols and Deamer, Cramer is concerned only with phenomenon of pH-induced transport of species, in this case carboxylic acids, across a liposome membrane. The only reference to elution of the encapsulated species is the statement

that "[T]he nonselective leakage of both fumaric and maleic acids depicted in Figure 4 at longer times is probably the result of vesicle rupture in response to the osmotic stress provided by the early selective transport of protonated fumaric acid." This, however, is observed while the pH gradient is still intact. There is no mention of what manner of buffer to use or to what happens to the incorporated chemical species once the gradient is destroyed as is the case in the current invention when the gradient solution is diluted under essentially pH neutral physiological conditions.

Again, the examiner is requested to reconsider and thereupon withdraw the rejection.

35 U.S.C. § 103 rejection of claim 1-12

The examiner has rejected claims 1-12 as being unpatentable over Nichols or Deamer in view of Cramer. While in the examiner's view, while Nichols, Deamer and Cramer do not teach the establishment of a pH gradient by addition If an acid, the examiner deems it to be within the "skill of the art of chemistry" that if an internal medium is basic one can only establish a gradient by the addition of an acidic substance. The examiner does find that Nichols, Deamer and Cramer all teach the loading of a chemical species into a liposome using a pH gradient.

Applicant traverses.

Applicant's response

Applicant does not dispute the examiner's contention that it is within the understanding of one or ordinary skill in the chemistry art that to establish a pH gradient when an internal medium is basic one should add an acid to the external medium. Applicant, however, contends that it is not within the ken of one or ordinary skill in the art to recognize that a chemical species can be maintained in the internal medium for a period of time after the loading gradient has been eliminated by using a membrane impermeable buffer. Thus, the present invention is not at all obvious.

The examiner is requested to reconsider and thereupon withdraw the rejection.

CONCLUSION

Applicant believes that based on the amendments to the claims herein and the above Remarks, this application is in condition for allowance and respectfully requests that it be passed to issue.

In addition, applicant requests a three month extension in time within which to file this response. The Commissioner is authorized to charge the amount due to SQUIRE, SANDERS & DEMPSEY Deposit Account No. 07-1850.

Respectfully submitted,

Date: May 29, 2008

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EXHIBIT H

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:	
MEHLHORN, Rolf Joachim	**Group Art Unit:** 1614
Serial No.: 10/759,222	**Examiner:** Weddington, Kevin E.
Filed: 20 January 2004	**Confirmation No.:** 6448
For: METHODS FOR LOADING LIPID LIKE VESICLES WITH DRUGS OR OTHER CHEMICALS	

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RESPONSE TO NON-FINAL OFFICE ACTION

With regard to the non-final office action mailed 5 September 2008, please take the following actions and consider the remarks below.

Terminal disclaimer

The examiner has maintained a judicially created double patenting rejection of the current application over U.S. Pat. No. 5,827,532.

Applicant's response

Applicant intended to provide a terminal disclaimer over the '532 patent with applicant's response to the 29 November 2007 office action by inadvertently failed to do so. The terminal disclaimer is submitted herewith.

CLAIMS

1. (PREVIOUSLY PRESENTED) A method of loading lipid-like vesicles, comprising:

forming lipid-like vesicles in a solution comprising an acidic buffer if the chemical species to be loaded is basic or a basic buffer if the chemical species to be loaded is acidic; wherein:

membranes of the formed lipid-like vesicles are impermeable to the buffer;

adjusting the pH of the solution exterior to the membranes of the lipid-like vesicles to a basic pH if the chemical species to be loaded is basic or to an acidic pH if the chemical species to be loaded is acidic;

adding a basic chemical species to the adjusted basic exterior solution or an acidic chemical species to the adjusted acidic exterior solution;

loading the chemical species into the vesicle; and

adjusting the exterior solution to a physiologically benign pH; wherein:

the chemical species is substantially maintained in the vesicle for at least one quarter hour after the adjustment of the exterior solution.

2. (PREVIOUSLY PRESENTED) A method of loading lipid-like vesicles having a membrane permeable to a chemical species to be loaded and for substantially maintaining the loaded chemical species within the vesicle for at least one-quarter hour following loading by inducing a pH gradient across the membrane, comprising:

(1) incorporating within the vesicle a buffer solution buffered to a selected acid or alkaline pH having a selected molarity and at least one selected pKa approximately equal to the selected buffer pH, the membrane being substantially impermeable to the buffer for at least one-quarter hour following loading of the chemical species;

(2) positioning the vesicles in a bulk solution having a selected pH; and

(3) providing the bulk solution with a chemical species having one or more selected acid pH responsive groups if the buffer is alkaline or one or more basic pH responsive groups if the buffer is acidic wherein the pH of the bulk solution is

at least 0.5, 0.3 or 0.2 of a pH unit higher than the pH of the buffer if the buffer is acidic and the chemical species has one, two, or three or more basic pH responsive groups, or the pH of the bulk solution is at least 0.5, 0.3 or 0.2 of a pH unit lower than the pH of the buffer if the buffer is basic and the chemical species has one, two or three or more acid pH responsive groups, the pH responsive groups of the chemical species having one or more acid pH responsive groups have a pKa that is generally lower than or equal to the pH of the bulk solution and generally higher than or equal to 3.5 and the pH responsive groups of the chemical species having one or more basic pH responsive groups have a pKa that is generally higher than or equal to the pH of the bulk solution and generally lower than or equal to 11.

3. (PREVIOUSLY PRESENTED) A method according to claim 2 wherein the pH responsive group or groups are acid pH responsive groups and the buffer has a pKa of about 10.

4. (PREVIOUSLY PRESENTED) A method according to claim 3 wherein the chemical species has a pKa of about 4-7.

5. (PREVIOUSLY PRESENTED) A method according to claim 4 wherein the pH responsive group is a carboxyl group.

6. (PREVIOUSLY PRESENTED) A method according to claim 2 wherein the pH responsive group or groups are basic pH responsive groups, and the buffer has a pKa in the range of about 5.

7. (PREVIOUSLY PRESENTED) A method according to claim 6 wherein the chemical species has a pKa from about 7-10.

8. (PREVIOUSLY PRESENTED) A method according to claim 7 wherein the pH responsive group is an amino group.

9. (PREVIOUSLY PRESENTED) A method according to claim 8 wherein the chemical species is an amine.

10. (PREVIOUSLY PRESENTED) A method according to claim 2 wherein the vesicle is prepared in the buffer and incorporates the buffer via mixing and sonication.

11. (PREVIOUSLY PRESENTED) A method according to claim 2 wherein the pH of the bulk solution is about 7.0 to about 7.8.

12. (PREVIOUSLY PRESENTED) A method according to claim 11 wherein the pH of the bulk solution is about 7.4.

13. (WITHDRAWN) The method of claim 2 wherein the chemical species is a drug.

14. (WITHDRAWN) A pharmaceutical preparation for administration in vivo to an animal comprising lipid-like vesicles prepared according to claim 1 wherein said chemical species is a drug.

15. (WITHDRAWN) A pharmaceutical preparation for parenteral administration in vivo to an animal comprising lipid-like vesicles prepared according to claim 2 wherein said chemical species is a drug, the osmolarity of the buffer is within the physiological range of the animal, the vesicles are suspended for administration in the bulk solution, and the pH of the bulk solution is physiologically benign.

16. (PREVIOUSLY PRESENTED) A kit for loading lipid-like vesicles having a membrane permeable to the chemical species to be loaded comprising:

(1) a first compartment having a first solution having membranous lipid-like vesicles incorporating a buffer buffered to a selected acid or basic pH, the buffer

having at least one selected pKa approximately equal to the selected buffer pH and a selected molarity and being substantially impermeable to the vesicle's membrane for at least one-quarter hour following loading of the chemical species and the first solution having a selected pH such that the stability of the vesicle and its buffer can be maintained for a period of at least one week at 4 °C.

(2) a second compartment, separate from the first compartment, having a second solution having a selected pH;

(3) a chemical species permeable to the vesicle having a selected pKa and one or more selected acid pH responsive groups if the buffer is basic or one or more basic pH responsive groups if the buffer is acidic, the chemical species being initially present in a selected one of two solutions with the second solution having a pH such that a mixture of the first and second solutions would have a pH at least 0.5, 0.3, or 0.2 of a pH unit higher than the pH of the buffer if the buffer is acidic and the chemical species has one, two, or three or more basic pH responsive groups at least 0.5, 0.3 or 0.2 of a pH unit lower than the pH of the buffer if the buffer is basic and the chemical species has one, two or three or more acid pH responsive groups, the pH responsive groups of the chemical species having one or more acid pH responsive groups have a pKa that is generally lower than or equal to the pH of the mixture of the first and second solution and generally higher than or equal to 3.5 and the pH responsive groups of the chemical species having one or more basic pH responsive groups have a pKa that is generally higher than or equal to the pH of the mixture of the first and second solutions and generally lower than or equal to 11.

17. (PREVIOUSLY PRESENTED) A kit as set forth in claim 16 wherein said chemical species is a drug.

18. (PREVIOUSLY PRESENTED) A kit as set forth in claim 17 wherein the mixture will have a pH that is physiologically benign in regard to the blood of a mammal.

19. (PREVIOUSLY PRESENTED) A kit as set forth in claim 18 further comprising means for parenterally delivering the mixture to a mammal in vivo.

20. (PREVIOUSLY PRESENTED) A kit for loading lipid-like vesicles having a membrane permeable to an acid or basic chemical species to be loaded comprising:

(1) a first compartment having a first solution having membranous lipid-like vesicles incorporating a buffer buffered to a selected basic pH if the chemical species to be loaded is an acid or acid pH if the species in a base, the buffer having a selected pKa and a selected molarity, the membrane being substantially impermeable to the buffer for at least one-quarter hour following loading of the chemical species, the first solution having a selected pH such that the stability of the vesicle and its buffer will be maintained for a period of at least one week at 4 °C;

(2) a second separate compartment having a first substance which when combined with the first solution will adjust the pH of the first solution so as to provide a predetermined pH gradient between the buffer within the vesicle and the pH adjusted first solution; and

(3) a third separate compartment having a second substance which when combined with the pH adjusted first solution will further change the pH of said solution to a physiologically benign value with regard to the blood of a mammal.

21. (PREVIOUSLY PRESENTED) A kit as set forth in claim 20 further comprising a selected chemical species.

22. (PREVIOUSLY PRESENTED) A kit as set forth in claim 21 wherein the selected chemical species is a drug.

23. (PREVIOUSLY PRESENTED) A kit as set forth in claim 22 further including a means for parentally delivering the vesicle solution having the physiologically benign adjusted pH to a mammal in vivo.

24. (WITHDRAWN) A method of detoxifying an animal suffering from an overdose of a chemical species with basic pH responsive groups comprising injecting the animal with a solution having a physiologically benign pH with respect to the animal, the solution having large volumes of liposomes having a buffer solution buffered to a pH generally lower than or equal to 5.4 and the buffer having at least one selected pKa and a selected molarity within the physiological range of the animal the liposomes being substantially impermeable to the buffer for at least one hour after injection.

25. (WITHDRAWN) A method for detoxifying an animal suffering from an overdose of a chemical species with acid pH responsive groups the chemical species being permeable to liposomes comprising: injecting the animal with a solution having a physiologically benign pH with respect to the animal, the solution having large volumes of liposomes having a buffer solution buffered to a pH generally higher than or equal to 9.4 and having a selected solarity and selected pKa, the liposomes being substantially impermeable to the buffer for at least one hour after injection.

26. (WITHDRAWN) A method of loading lipid-like vesicles having a membrane permeable to a chemical species to be loaded and substantially maintaining the loaded chemical species by inducing a pH gradient across the membrane within the vesicle for at least one-quarter hour following loading, comprising:

(1) incorporating within the vesicle a buffer solution buffered to a selected acid or alkaline pH and having a selected molarity and at least one selected pKa, the membrane being substantially impermeable to the buffer for at least one-quarter hour following loading of the chemical species;

(2) positioning the vesicles in a bulk solution having a selected pH of either 0.5 to 3 pH units lower or pH units higher than the pH of the buffer thereby establishing a transmembrane electrical potential and a positive charge inside the vesicle if the pH outside the vesicle is more acid than inside or a negative charge inside the cell if the pH outside the call is more basic than inside;

(3) providing in the bulk solution a chemical species having hydrophobic negatively-charged ions if the membrane charge within the vesicle is positive or

hydrophobic positively charged ions if the membrane charge within the vesicle is negative.

REMARKS

Claims 1-26 are currently before the examiner. Claims 13-15 and 24-26 have been withdrawn by the examiner as, in the examiner's view, being drawn to separate and distinct invention. Applicant traversed the withdrawal, which the examiner did not deem persuasive. Claims 1-12 and 16-23 stand rejected.

Double patenting

The examiner has maintained the non-statutory double patenting rejection of claims 16-23 over claims 1-7 of U.S. Pat. No. 5,762,957. The examiner states that the inventions are not patently distinct for reasons of record as set forth in the 29 November 2007 office action.

The examiner augments the previous grounds for the double patenting rejection by stating that applicant's remarks are non-persuasive regarding the differences between the current kits and those of the '957 patent. In the examiner's view, the current kit and the '957 kit "are claiming the same components in both kits" and therefore would produce the same results.

Applicant traverses.

Applicant's response

Applicant maintains and incorporates herein the rationale presented in the response to the 29 November 2007 office action for finding the kits of the current application and those of the '957 patent patently distinct are valid. That is,

> The kit of the current claims require a first compartment containing an acidic or basic buffer solution both within and outside lipid-like vesicles, wherein the vesicles are substantially impermeable to the buffer for at least one-quarter hour following loading of the chemical species and, further, the first solution has a pH selected such that the stability of the vesicle and its buffer can be maintained for a period of at least one week at 4 °C. The current claims also require a second compartment having a second solution at a selected pH wherein when the first and second solutions are mixed the resulting solution has a pH that is 0.5, 0.3 or 0.2 pH units above the pH of the buffer in the liposomes if the buffer is acidic or 0.5, 0.3 or 0.2 pH units below the pH of the buffer in the liposomes if the buffer is basic.

> The patented kits, on the other hand, make no mention whatsoever as to the duration of the impermeability of its liposome membranes to the buffer or to the selection of a first solution pH so as to result in stable vesicles and buffers for at least one week. Further, the patented invention says nothing about the pH of the combined first and second solutions. Thus the patented kit requires only acids and bases and does not specify any particular pH while the current application does. The difference, i.e., from no preference to the express limitations of the current invention are not obvious.

Applicant emphasizes that the components of the kits are not the same. The current kits require a very specific relationship between the pHs of the first and second solutions which is nowhere evident in the kit components of the '957 patent.

Further, the kits of the current invention require that the pH of the first solution be such that "the stability of the vesicle and its buffer can be maintained for a period of at least one week at 4 °C, which element is completely absent from the kits of the '957 patent. The mere fact that the possibility of such a composition is disclosed in the '957 patent specification is of no import. The '957 kit claims do not claim such. To suggest that the kits of the '958 patent would "produce the same results" without virtually any of the specific limitations of the current kits would be to incorporate limitations from the specification into the claims, which the examiner is well aware is impermissible.

The examiner is requested to reconsider and thereupon withdraw the terminal disclaimer over U.S. Pat. No. 5,762,957.

35 U.S.C. §112, first paragraph rejection of claims 1-8, 10-12 and 16-23

The examiner has rejected claims 1-8, 10-12 and 16-23 under §112, first paragraph, because, in the examiner's view, the specification "fails to provide sufficient written bases of any of the agents demonstrating wherein possession of use of the broad terms: a chemical species, an amino group, an amine, a drug, a first substance, and a second substance."

Applicant traverses.

Applicant's response

Applicant is not laying claim to certain chemical species, amines, drugs and the like. Rather, applicant is claiming a general purpose method of loading acid and basic

chemical species into lipid-like vesicles. Contrary to the examiner's position, there is no authority stating that a single example is insufficient to support a general method claim absent compelling reasons to lead one of ordinary skill in the art to believe that the method would not work with most if not all other species of the exemplified substance. Specifically, the term "chemical species" is not used in the claim anywhere without ultimately being modified for the purpose or the claim as being an acidic or basic chemical species or a chemical species containing an "acid pH responsive" or a "basic pH responsive" group. This is a sufficient description to permit those skilled in the art to immediately envision the types of acidic/basic chemical species or acid/basic pH responsive groups since pH is a fundamental concept of organic chemistry known to all practitioners of the organic chemical art. The chemical species are further defined in the specification at page 3, paragraph [0023] and page 4, paragraph [0030]:

> [0023] ... The term chemical species having one or more selected acid or basic pH responsive groups is also used broadly to indicate any chemical or drug having acid or basic groups, properties or functions such as, but not limited to amine or carboxyl groups. Other substances such as imidazoles and barbituric acid derivatives may also be used. The term also includes any chemical that has desired chemical or therapeutic properties that will not be sufficiently altered by attachment of such pH responsive groups. ...
>
> [0030] ... The chemical [species] loading rate will depend on the pKa and will be complete within less tha minute for low molecular weigh (MW less than 500) amine chemicals with pKs's less than 10 and having no charge or strongly polar groups other than the amino group. Analogously, weak acids [chemical species] having pKas greater than 4 will accumulate in the liposomes in about one minute, unless they bear strong polar groups other than their carboxyls.

Applicant believes that no more is required to sufficiently explicate what is meant by "chemical species."

"Amino" and "amine" need no further definition. Those skilled in the art will immediately understand these terms to refer to a –NRR' group wherein R and R' are independently hydrogen, alkyl and/or aryl and to compounds containing such group and would likewise immediately know that the group or compound will have a basic pH.

Such commonly-known terms do not require definition in an application unless the usage there is different from what those skilled in the art would expect. Such is not the case here. Example 1 provides a specific basic entity, Tempamine, which would be immediately recognized by those skilled in the art to be an "amine" and to contain one or more "amino" groups, to demonstrate the fundamental operation of the claimed method. There is nothing to suggest that other amines would not work.

With regard to drugs, more than sufficient examples are provided. In paragraph [0023], it is stated:

> [0023] ... The terms chemical species and "drugs" include but are not limited to, such substances as chemicals, drugs for chemotherapy and immunosuppression, membrane permeable peptide toxins and hormones. Examples of drugs having molecules having basic properties are vincristine, doxorubicin, streptomycin, chloroquine and daunorubicin. Examples of drugs having molecules having acid properties are derivatives of methotrexate, daunomycin, penicillin, p-amino salicylic acid and salicylic acid derivates.

Methotrexate is exemplified in Example 4. Applicant maintains that no more description is necessary or warranted. The method is intended to be broadly applicable to any chemical species having the requisite properties and there is nothing to suggest that its applicability is any less expansive than claimed and described.

35 U.S.C. § 103 rejection of claims 1-12

The examiner has rejected claim 1-12 as being unpatentable over Nichols, etal, or Deamer, et al, or Cramer, et al. The examiner states that each of these references teaches the concept of loading a chemical species into the liposomes using a pH gradient and that it would have been obvious to one skilled in the art to load any drug with the expectation of similar loading since applicant has not demonstrated side-by-side comparison of the prior art's liposome loading versus the present application's liposome loading.

Applicant traverses.

Applicant's response

As applicant pointed out in his response to the 19 November 2007 office action to the §102 rejections over Nichols, Deamer and Cramer, applicant does not dispute that

using pH gradients to load vesicles. Applicant pointed out, and reiterates here, that nowhere in Nichols, Deamer or Cramer is the concept taught or so much as suggested of using membrane impermeable buffers to maintain loading after the gradient is destroyed. Since none of the cited references individually teaches or suggests such, clearly the combination of them cannot do so.

The examiner is requested to reconsider and thereupon withdraw the rejection.

CONCLUSION

Applicant believes that based on the above Remarks, this application is in condition for allowance and respectfully requests that it be passed to issue.

In addition, applicant requests a one month extension in time within which to file this response. The Commissioner is authorized to charge the fee due to SQUIRE, SANDERS & DEMPSEY Deposit Account No. 07-1850.

Dated: January 5, 2009

Respectfully submitted,

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RELATED PROCEEDINGS APPENDIX

There are no related proceedings.